

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
13 January 2005 (13.01.2005)

PCT

(10) International Publication Number  
**WO 2005/003291 A2**

(51) International Patent Classification<sup>7</sup>: C12N

(21) International Application Number: PCT/US2003/032218

(22) International Filing Date: 14 October 2003 (14.10.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
10/272,509 16 October 2002 (16.10.2002) US

(71) Applicant: **BOARD OF REGENTS OF THE UNIVERSITY OF TEXAS SYSTEM** [US/US]; 201 West 7th Street, Austin, TX 78701 (US).

(72) Inventors: **GORENSTEIN, David, G.**; 3922 Crown Ridge Court, Houston, TX 77059 (US). **YANG, Xian**,

Bin; 515 First Street, Galveston, TX 77550 (US). **LUXON, Bruce, A.**; 18 Cedar Lawn Circle, Galveston, TX 77551 (US). **HERZOG, Norbert**; 2008 Schulte Lane, Friendswood, TX 77546 (US).

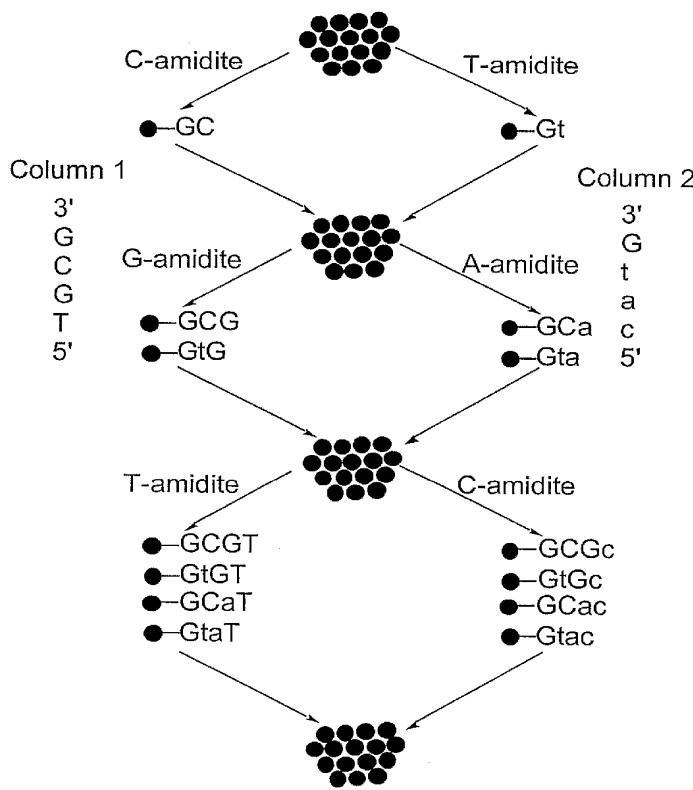
(74) Agents: **FLORES, Edwin** et al.; Chalker Flores, LLP, 12700 Park Central Drive, Suite 455, Dallas, TX 75251 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

*[Continued on next page]*

(54) Title: BEAD BOUND COMBINATORIAL OLIGONUCLEOSIDE PHOSPHOROTHIOATE AND PHOSPHODITHIOATE APTAMER LIBRARIES



(57) Abstract: The present invention includes composition and methods for making and using a combinatorial library having two or more beads, wherein attached to each bead is a unique nucleic acid aptamer that have disposed thereon a unique sequence. The library aptamers may be attached covalently to the one or more beads, which may be polystyrene beads. The aptamers may include phosphorothioate, phosphordithioate and/or methylphosphonate linkages and may be single or double stranded DNA, RNA, or even PNAs.



European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**Published:**

- *without international search report and to be republished upon receipt of that report*

**BEAD BOUND COMBINATORIAL OLIGONUCLEOSIDE PHOSPHOROTHIOATE  
AND PHOSPHORODITHIOATE APTAMER LIBRARIES**

**TECHNICAL FIELD OF THE INVENTION**

The present invention relates in general to the field of aptamer libraries, and more particularly, to enhancing availability and use of aptamers for screening, including high-throughput screening, of primary or secondary target molecules by using thioated aptamers bound to a substrate.

**BACKGROUND OF THE INVENTION**

This work was supported by the following United States Government grants DARPA (9624-107 FP), NIH (AI27744) and NIEHS (ES06676). Without limiting the scope of the invention, its background is described in connection with oligonucleotide agents and with methods for the isolation of sequences that are bound by nucleic acid binding molecules and the like.

Virtually all organisms have nuclease enzymes that degrade rapidly foreign DNA as an important *in vivo* defense mechanism. The use, therefore, of normal oligonucleotides as diagnostic or therapeutic agents in the presence of most bodily fluids or tissue samples is generally precluded. It has been shown, however, that phosphoromonothioate or phosphorodithioate modifications of the DNA backbone in oligonucleotides can impart both nuclease resistance and enhance the affinity for target molecules, such as for example the transcriptional regulating protein NF- $\kappa$ B. Thus, from the foregoing, it is apparent there is a need in the art for methods for generating aptamers that have enhanced binding affinity for a target molecule, as well as retained specificity. Also needed are ways to identify and quantify in detail the mechanisms by which aptamers interact with target molecules.

Current DNA array technology is problematic in that it is focused on the identification and quantification of a single mRNA species, and does not provide information on the more relevant level of functional protein expression and in particular protein-protein interactions such as between heterodimers and homodimers. Although microarrays have been used for detecting the proteome, most of these are based on antibodies or normal backbone aptamers.

Synthetic phosphodiester-modified oligonucleotides such as phosphorothioate oligonucleotide (S-ODN) and phosphorodithioate oligonucleotide (S<sub>2</sub>-ODN) analogues have increased nuclease resistance and may bind to proteins with enhanced affinity. Unfortunately, ODNs possessing high fractions of phosphorothioate or phosphorodithioate linkages may lose some of their

specificity and are “stickier” towards proteins in general than normal phosphate esters, an effect often attributed to non-specific interactions. The recognition of nucleic acid sequences by proteins involves specific sidechain and backbone interactions with both the nucleic acid bases as well as the phosphate ester backbone, effects which may be disrupted by the non-specific 5 interactions caused with S-ODN and S<sub>2</sub>-ODN analogues.

Gorenstein, et al., U.S. Patent No. 6,423,493, have taken advantage of this “stickiness” to enhance the affinity of S-ODN and S<sub>2</sub>-ODN agents for a protein target. A screening method was developed to optimize the total number of phosphorothioate or phosphorodithioate linkages that: decreased non-specific binding to the protein target while enhancing only the specific favorable 10 interactions with the target protein.

Other advances in combinatorial chemistry allow construction and screening of large random sequence nucleic acid “aptamer” libraries (e.g., Ellington, A.D. and Szostak, J.W. (1990) *In vitro* selection of RNA molecules that bind specific ligands. *Nature*, 346, 818-822); targeting proteins (e.g., Bock, L.C., et al., (1992) Selection of single-stranded DNA molecules that bind and inhibit 15 human thrombin. *Nature*, 355, 564-566.); and other molecules (Koizumi, M. and Breaker, R.R. (2000) Molecular recognition of cAMP by an RNA aptamer. *Biochemistry*, 39, 8983-8992; Gold, L., et al. (1997) SELEX and the evolution of genomes. *Curr. Opin. Genetic. Dev.*, 7, 848-851.; and Ye, X., et al. (1996) Deep penetration of an  $\alpha$ -helix into the widened RNA major groove in the HIV-1 Rev peptide-RNA aptamer complex. *Nat. Struct. Biol.*, 3, 1026-1033.).

20 The identification of specific S-ODN and S<sub>2</sub>-ODN aptamers (“thioaptamers”) that bind proteins based upon *in vitro* combinatorial selection methods, however, is limited to substrates only accepted by polymerases required for reamplification of selected libraries by the polymerase chain reaction (PCR). One disadvantage of using the polymerization of substituted nucleoside 5'-triphosphates into ODN aptamers are the restrictions on the choice of P-chirality by the 25 enzymatic stereospecificity (1). For example, it is known that [S<sub>P</sub>]-diastereoisomers of dNTP( $\alpha$ S) in Taq-catalyzed polymerization solely yield [R<sub>P</sub>]-phosphorothioate stereoisomers (Eckstein, F. (1985) Nucleoside phosphorothioates. *Annu. Rev. Biochem.*, 54, 367-402.). Therefore, using current methods it is not possible to select [S<sub>P</sub>]-phosphorothioate stereoisomers along with achiral S<sub>2</sub>-ODN analogous since both [Rp]-diastereoisomers of dNTP( $\alpha$ S) and 30 nucleoside dNTP( $\alpha$ S<sub>2</sub>) are not substrates of polymerases. Additionally, these *in vitro* combinatorial selection methods require many iterative cycles of selection and reamplification of the bound remaining members of the library by the PCR, which are quite time consuming.

What is needed are compositions and methods that permit the isolation of, e.g., individual aptamer:protein complexes without the need for repeated iterative cycles of selection and reamplification of likely binding targets. Also needed are compositions, methods and systems that permit the creation, isolation, sequencing and characterization of making [S<sub>P</sub>]-

5 phosphorothioate stereoisomers along with achiral S<sub>2</sub>-ODN analogs using, e.g., [R<sub>P</sub>]-diastereoisomers of dNTP(αS) and nucleoside dNTP(αS<sub>2</sub>). Also needed are methods for creating libraries that permit not only the isolation of a primary aptamer:protein target, but the isolation of protein(s) that may interact with the aptamer:protein target, so called secondary interactions.

10

#### SUMMARY OF THE INVENTION

The present invention addressed the problems in the prior art by developing composition and methods for making and using a combinatorial library in which each substrate, e.g., a bead, has attached thereto a unique ODN sequence. More particularly, the one-bead, one-ODN library of the present invention includes two or more beads, wherein attached to each bead is a unique

15 nucleic acid aptamer that have disposed thereon a unique sequence. The bead library aptamers may be attached covalently to the one or more beads, which may be polystyrene beads. The aptamers may include phosphorothioate, phosphorodithioate and/or methylphosphonate linkages and may be single or double stranded DNA, RNA or even PNAs.

The ODNs attached to the substrate or bead or the present invention may also include one or

20 more predetermined nucleic acid sequences, e.g., having at least 10, 12, 16, or more bases. The predetermined sequence may be a 5' nucleic acid sequence, a 3' nucleic acid sequence, and a 5' and a 3' nucleic acid sequence to the ODN. In one embodiment the ODN is attached to, e.g., a polystyrene/polydivinyl benzene copolymer bead with, e.g., a hexaethyleneglycol linker. The aptamers may be isosteric, isopolar and/or achiral. The aptamers may further include a

25 detectable marker, e.g., a colorimetric agent such as a fluorophor. The detectable marker may be attached to the 5' end, the 3' end or internally within the aptamers. The aptamers of the present invention may be single or double stranded.

In another embodiment of the present invention the one-bead, one-ODN combinatorial library includes two or more beads, attached to each bead is a unique aptamer that has a single unique

30 sequence, and each unique aptamer includes a mix of modified and unmodified nucleotides. In one embodiment the aptamer is double stranded and the modifications to each strand is unique and does not mirror the modifications to the complementary strand.

Yet another embodiment of the present invention is an ODN library in which a library substrate has a surface and attached to the substrate library are the individual beads of a one-bead, one-ODN bead library. The library substrate may be, e.g., a bead, a chip, a chip that includes a capacitance-coupled detector, a photolithographically etched microwell plate to contain beads 5 (“Texas tongue”) or even a glass slide.

The present invention also includes a method of making a combinatorial library including the steps of: attaching a single base to a first bead in a first column and a single thio-modified base (or a different nucleoside or modified nucleoside monomeric unit) to a second bead in a second 10 column and mixing the first and second beads. Next, the mixed first and second beads are split into the first and second columns, a new base is added to the separated beads in each of the first and second columns and the steps of mixing the beads, splitting the beads and adding a new base in each of the first and second columns are repeated until the library is complete. The aptamers may be converted to double-stranded aptamers using, e.g., a DNA polymerase I Klenow fragment.

15 Yet another embodiment of the present invention is a method for detecting nucleic acid-protein interactions by mixing a one-bead, one-ODN combinatorial library with one or more proteins and detecting the binding of the protein to one or more beads of the one-bead, one-ODN combinatorial library. The reaction is carried-out generally under conditions that permit the binding of a second protein to the protein bound to the one or more beads. Also, the sequence on 20 the bead may be determined by isolating the bead and sequencing the unique aptamer bound to the bead, which may be done, e.g., to compare the level of protein bound to one or more of the beads a control and a test sample. The level of protein bound to the one or more beads may also be from a control and a patient sample.

Another method of the present invention is a way to identify proteins differentially expressed in 25 a sample, by mixing a one-bead, one-ODN aptamer bead library where the sample has been labeled with a first dye and with a control labeled with a second dye under conditions that allow binding followed by sorting the bead library and comparing the relative levels of each of the first and second dyes on each bead. In this assay the differences in the level of the first or second dye are used to determine the level of binding of the sample and the control to the beads in the bead 30 library. The beads may be sorted by a flow cytometer or even manually. For sample evaluation the present method may also include the step of isolating the one or more beads and determining the characteristics of the bound material by SELDI-MS. The first and second dyes are

fluorescent dyes, e.g., cy3 and cy5. The method may also include placing the library of beads onto a substrate. Using the methods of the present invention, it is also possible to increase the amount of dyes or other chromo or fluorophores to achieve sorting of the beads in the bead library by a variable selection criteria, e.g., a low, medium and/or high signal selection criteria.

5 According to one embodiment of the present invention, the modified nucleotide aptamer can contain a phosphoromonothioate or phosphorodithioate (“phosphorothioates”) and can be selected from the group consisting of dATP(αS), dTTP(αS), dCTP(αS) and dGTP(αS). In another embodiment of the present invention, no more than three adjacent phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups. In yet another embodiment of the present invention, at least a portion of non-adjacent dA, dC, dG, or dT phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups. In yet another embodiment of the present invention, all of the non-adjacent dA, dC, dG, or dT phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups. In yet another embodiment of the present invention, all of the non-adjacent dA, dC, dG, and dT phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups. In still another embodiment of the present invention, substantially all non-adjacent phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups.

In accordance with another embodiment of the present invention, the target molecule or portion thereof is NF-κB. In accordance with another embodiment of the present invention, the aptamer is selected to bind NF-κB or constituents thereof and is essentially homologous to the sequences of oligonucleotides that bind NF-κB but one or more nucleotides have at least one thiophosphate or dithiophosphate group. In yet another embodiment of the present invention, the aptamer is selected to bind NF-κB or constituents thereof and wherein at least one nucleotide is an achiral thiophosphate or a dithiophosphate. In yet another embodiment of the present invention, the aptamer is selected to bind NF-κB or constituents thereof and wherein at least one nucleotide is an achiral thiophosphate or a dithiophosphate.

In yet another embodiment of the present invention, between 1 and 6 of the phosphate sites of the modified nucleotide aptamer are dithiophosphates. In another embodiment of the present invention, the modified nucleotide aptamer contains 6 dithioate linkages. In one embodiment of the invention, the detection method is selected colorimetric, chemiluminescent, fluorescent, radioactive, mass spectrometric, capacitance coupled electrical, Biacor or combinations thereof. The apparatus of the present invention may further include aptamer libraries containing multiple

different but related members. In one embodiment of the present invention, the substrate for the library is selected from the group consisting of beads, membranes, glass, and combinations thereof. The substrate may even be a microarray of beads or other substrates.

In one embodiment of the present invention, an apparatus for monitoring biological interactions 5 on the surface of the substrate, e.g., a bead library, is disclosed. The library can include a substrate, a modified nucleotide aptamer attached to the substrate, and a target protein or portion thereof. The target protein or portion thereof may be complexed with the modified nucleotide aptamer under conditions sufficient to allow complexation between the aptamer and the target protein or portion thereof. The modified nucleotide aptamer may include an oligonucleotide 10 having a desired binding efficiency for a target protein or portion thereof.

According to one embodiment of the present invention, the modified nucleotide aptamer is selected by the steps of: attaching a first base to a bead or other substrate; synthesizing a random phosphodiester oligonucleotide combinatorial library wherein constituent oligonucleotides comprise at least a set of 5' and 3' PCR primer nucleotide sequences flanking a randomized 15 nucleotide sequence using a split synthesis method, adding the next base, wherein at least a portion of at least one of the nucleotides in the mix is thiophosphate-modified, to form a partially thiophosphate-modified oligonucleotide combinatorial library, and repeating the steps of adding a base that is either thiophosphate-modified or a phosphodiester linked nucleotide iteratively a population of sequences is obtained.

20 According to one embodiment of the present invention, the steps in which any number of beads are included in a column that adds a thiophosphate-modified base is limited so that no more than three adjacent phosphate sites of the modified nucleotide aptamer are replaced with phosphorothioate groups.

The present thioaptamer methodology may also provide a library and method of use for 25 identifying aptamers that are improvement over existing antisense or "decoy" oligonucleotides because of their stereochemical purity. Chemically synthesized phosphorothioates may be a diastereomeric mixture with  $2^n$  stereoisomers with n being the number of nucleotides in the molecule. These preparations are unsuitable for use in humans because only a small fraction of the stereoisomers will have useful activity and the remaining could have potential adverse 30 effects. In contrast, enzymatically synthesized oligonucleotides are stereochemically pure due to the chirality of polymerase active sites. Inversion of configuration is believed to proceed from R<sub>p</sub> to S<sub>p</sub> during incorporation of dNMP $\alpha$ S into the DNA chain. These chiral

phosphoromonothioates can be incorporated into the complementary strand of duplexes using polymerases and a mix of normal and at least one, but no more than three of dATP( $\alpha$ S), dTTP( $\alpha$ S), dCTP( $\alpha$ S) and dGTP( $\alpha$ S) (or NTP( $\alpha$ S)'s for RNA thioaptamers) as described in (Gorenstein, D.G., et al., U.S. Patent, 6,423,493). The present dithiophosphate aptamers are free 5 from diastereomeric mixtures.

The present inventors have developed chemically synthesized combinatorial libraries of unmodified or modified nucleic acids and methods for using the same, to select rapidly oligonucleotides that bind to target biomolecules, e.g., proteins. The present inventors used a split synthesis methodology to create one-bead one-S-ODN and one-bead one-S<sub>2</sub>-ODN libraries.

10 Binding and selection of specific beads to the transcription factor NF- $\kappa$ B p50/p50 protein were demonstrated. Sequencing both the nucleic acid bases and the positions of any 3'-O-thioate/dithioate linkages was carried out by using a novel PCR-based identification tag of the selected beads. The use of a PCR-based identification tag allowed the rapid and convenient identification of S-ODNs or S<sub>2</sub>-ODNs that bound to proteins. Phosphorothioate oligonucleotides 15 (S-ODN) or phosphorodithioate oligonucleotide (S<sub>2</sub>-ODNs) with sulfurs replacing one or both of the non-bridging phosphate oxygens were shown to bind to proteins more tightly than unmodified oligonucleotides, and have the potential to be used as diagnostic reagents and therapeutics.

20 The present invention is a one-bead, one-compound library made by using a split synthesis method to create an alternative to *in vitro* combinatorial selection methods. One-bead library systems have been used for organic molecules (Felder, E.R. (1999) Resins, Linkers And Reactions For Solid-Phase Synthesis Of Organic Libraries. In Miertus, S. (ed.), *In Combinatorial Chemistry and Technology, Principles, Methods and Applications*. Marcel Dekker, Inc., NY, pp. 35-51.); peptides (Lam, K.S., et al., (1991) A new type of synthetic peptide library for 25 identifying ligand-binding activity. *Nature*, 354, 82-84; Lam, K.S., et al., (1997) The "one-bead-one-compound" combinatorial library method. *Chem. Rev.*, 97, 411-448; Lam, K.S. (1995) Synthetic peptide libraries. In Molecular Biology and Biotechnology: A Comprehensive Desk Reference. Meyer, R.A. (ed.) p.880. VCH Publisher:NY.); and oligosaccharide libraries (Zhu,T., and Boom,G.J. (1998) A two-directional approach for the solid-phase synthesis of trisaccharide 30 libraries. *Angew. Chem. Int. Ed.*, 37, 1898-1900.; Liang, R., et al., (1996) Parallel synthesis and screening of a solid phase carbohydrate library. *Science*, 274, 1520-1522.; Hilaire, P.M.St. and Meldal, M. (2000) Glycopeptide and oligosaccharide libraries. *Angew. Chem. Int. Ed.*, 39, 1162-1179.). The present invention is the first to demonstrate a one-bead one-oligonucleotide (one-

ODN) (e.g., S-ODN, S<sub>2</sub>-ODN or RNA) combinatorial library selection methodology used to identifying a specific oligonucleotide aptamer that binds to specific proteins or other molecules.

Furthermore, the present invention may use S<sub>2</sub>-ODN reagents with sulfurs replacing both of the non-bridging phosphate oxygens that are isosteric and isopolar with the normal phosphorodiester  
5 and are particularly advantageous for binding and screening. Importantly, S<sub>2</sub>-ODNs are achiral about the dithiophosphate center, which eliminates problems associated with diastereomeric mixtures generally obtained for the chemically synthesized S-ODN. The split synthesis approach described herein was used for the construction of S-ODN, S<sub>2</sub>-ODN and RNA bead-based thioaptamer libraries. For example, specific S-ODNs and S<sub>2</sub>-ODNs were identified by  
10 screening of the libraries against a transcription factor NF-κB p50 or p65 heterodimers. Sequencing of both the nucleic acid bases and the positions of any 3'-O-thioate/dithioate linkages was carried out by using a novel PCR-based identification tag of the selected beads.

The controlled thiolation methodology and the libraries made thereby are applicable to the design of specific, nuclease resistant aptamers to virtually any target, but not limited to, amino  
15 acids, peptides, polypeptides (proteins), glycoproteins, carbohydrates, nucleotides and derivatives thereof, cofactors, antibiotics, toxins, and small organic molecules including, dyes, theophylline and dopamine. The nuclease resistant aptamers may be targeted against viruses, bacteria, parasites, neoplastic cells and the like. It is within the scope of this invention, that the instant thioaptamers encompass further modifications to increase stability and specificity  
20 including, for example, disulfide crosslinking. It is further contemplated and within the scope of this invention that the instant thioaptamers encompass further modifications including, for example, radiolabeling and/or conjugation with reporter groups, such as biotin or fluorescein, or other functional or detectable groups for use in *in vitro* and *in vivo* diagnostics and therapeutics.

The present invention further provides the application of this methodology to the generation of  
25 novel thiolated aptamer libraries specific for nuclear factors such as, for example, NF-IL6 and NF-κB. By taking advantage of cognate binding motifs, the library may be focused to reduce library size, while also taking into account the required diversity of ODN species. The NF-κB /Rel family of transcription factors are key mediators of immune and acute phase responses, apoptosis, cell proliferation and differentiation. The NF-κB/Rel transcription factors are also key  
30 transcriptional regulators acting on a multitude of human and pathogen genes, including HIV-1.

The present structure-based dithiophosphate and combinatorial monothiophosphate library system provides for the identification of aptamers that have high specificity, and high affinity for

DNA binding proteins, for example, a single NF-κB heterodimer, in a cellular extract in a rapid, single well assay followed by, e.g., rapid sorting of the bead using a flow-cytometer calibrated to the bead size(s) used to make the library. The present invention encompasses the development of separate aptamers targeting any one of the 15 possible combinations of, e.g., 5 homo- and 5 hetero-dimers of the 5 different forms of NF-κB/Rel. NF-κB/Rel proteins are not only capable of transactivation (heterodimers that include NF-κB RelA(p65), c-Rel, RelA, but also repression (homodimers of NF-κB p50 or p52).

The one-bead, one-ODN libraries of the present invention may be used to study and in treatment of the many diseases in which transcription factors play a critical role in gene activation, 10 especially acute phase response and inflammatory response. These diseases include, but are not limited to: bacterial pathogenesis (toxic shock, sepsis), rheumatoid arthritis, Crohn's disease, generalized inflammatory bowel disease, hemorrhagic fevers, autoimmune disorders, asthma, cardio-pulmonary disease, atherosclerosis, asbestos lung diseases, Hodgkin's disease, prostate cancer, ventilator induced lung injury, general cancer, AIDS, human cutaneous T cell lymphoma, 15 lymphoid malignancies, HTLV-1 induced adult T-cell leukemia, atherosclerosis, cytomegalovirus, herpes simplex virus, JCV, SV-40, rhinovirus, influenza, neurological disorders and lymphomas.

Single-stranded nucleic acids are also known to exhibit unique structures. The best documented single-stranded nucleic acid structures are single-stranded RNA. Single-stranded DNA can also 20 adopt unique structures. The present invention is applicable to the selection of single-stranded phosphorothioate aptamers of either RNA or DNA. Such single-stranded aptamers are applicable to both DNA (i.e., cell surface receptors, cytokines, etc.) and non-DNA binding proteins.

It is contemplated that the present methods and procedures may be scaled-up as would be 25 necessary for high throughput thioaptamer screening and selection. For example, 6, 12, 48, 96 and 384 well microtiter plates may be used to select pools of aptamers in the one-bead, one-ODN librart to a number of different proteins under numerous conditions, e.g., for use with in conjunction with a plate reader or even an ELISA assay.

According to one embodiment of the present invention, the one-bead, one-ODN library may be 30 employed that discriminates among 100's or even 1000's of proteins and particularly protein-protein complexes in the cell, simultaneously. Although the rate of dissociation and

equilibration may vary, the rate of dissociation and equilibration of the different complexes typically is slow relative to the assay time, which is not a problem for NF- $\kappa$ B/Rel.

An ODN library that includes a substrate for a library, the substrate having at least one surface. Attached to the surface of the library, is a library of one-bead, one-combinatorial library ODN 5 beads attached to the library substrate surface, thus making a library of libraries. The substrate may be, e.g., a chip, glass, glass slide, quartz, a gold surface, a surface plasmon resonance detector, a photolithographically etched micromachined microwell chip or "Texas tongue" and the like. The substrate may even be a capacitance coupled detector or other like electromagnetic, 10 magnetic, electrical or optical detector. In one embodiment, the ODN library or libraries is made by attaching a single base to a first set of beads in a first column and attaching a mixture of unmodified or modified nucleotides, which as used herein includes unmodified bases with modified phosphate backbone(s) (i.e., sugar phosphate analogs) to a second set of beads in a second column. Next, the first and second set of beads from the first and second columns are 15 mixed and then again into the first and second columns. A new base or a mixture of unmodified or modified bases or phosphate backbone analogs are added to the mixed beads in each of the first and second columns. These steps are repeated until the library is complete. In the final library of libraries, each of the oligonucleotides on each bead is now a combinatorial library and each unique oligonucleotide on each bead may include unmodified or a mix of modified and unmodified nucleotides

20 BRIEF DESCRIPTION OF THE DRAWINGS

For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

25 Figure 1 is a flow diagram of one example for the solid-phase synthesis of a one-bead one-S/S<sub>2</sub>-ODN library;

Figures 2A – 2C shown a portion of the beads are viewed under both light and fluorescence microscope; and

Figures 3A-3D, and as a demonstration of the use of the one-bead, one-ODN:protein system and for dual color bead sorting.

DETAILED DESCRIPTION OF THE INVENTION

While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific 5 embodiments discussed herein are merely illustrative of specific ways to make and use the invention and do not delimit the scope of the invention.

To facilitate the understanding of this invention, a number of terms are defined below. Terms defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the present invention. Terms such as "a", "an" and "the" are not intended to refer to 10 only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not delimit the invention, except as outlined in the claims.

As used herein, "synthesizing" of a random combinatorial library refers to chemical methods known in the art of generating a desired sequence of nucleotides including where the desired 15 sequence is random. Typically in the art, such sequences are produced in automated DNA synthesizers programmed to the desired sequence. Such programming can include combinations of defined sequences and random nucleotides.

"Random combinatorial oligonucleotide library" means a large number of oligonucleotides of different sequence where the insertion of a given base at given place in the sequence is random. 20 "PCR primer nucleotide sequence" refers to a defined sequence of nucleotides forming an oligonucleotide which is used to anneal to a homologous or closely related sequence in order form the double strand required to initiate elongation using a polymerase enzyme. "Amplifying" means duplicating a sequence one or more times. Relative to a library, amplifying refers to *en masse* duplication of at least a majority of individual members of the library.

25 As used herein, "thiophosphate" or "phosphorothioate" are used interchangeably to refer analogues of DNA or RNA having sulphur in place of one or more of the non-bridging oxygens bound to the phosphorus. Monothiophosphates or phosphoromonothioates [ $\alpha$ S] have only one sulfur and are thus chiral around the phosphorus center. Dithiophosphates are substituted at both oxygens and are thus achiral. Phosphoromonothioate nucleotides are commercially available or 30 can be synthesized by several different methods known in the art. Chemistry for synthesis of the phosphorodithioates has been developed by one of the present inventors as set forth in U. S.

Patent #5,218,088 (issued to Gorenstein, D.G. and Farschtschi, N., June 8, 1993 for a Process for Preparing Dithiophosphate Oligonucleotide Analogs via Nucleoside Thiophosphoramidite Intermediates), relevant portions incorporated herein by reference.

“Modified” is used herein to describe oligonucleotides or libraries in which one or more of the 5 four constituent nucleotide bases of an oligonucleotide are analogues or esters of nucleotides normally comprising DNA or RNA backbones and wherein such modification confers increased nuclease resistance. Thiophosphate nucleotides are an example of modified nucleotides. “Phosphodiester oligonucleotide” means a chemically normal (unmodified) RNA or DNA 10 oligonucleotide. Amplifying “enzymatically” refers to duplication of the oligonucleotide using a nucleotide polymerase enzyme such as DNA or RNA polymerase. Where amplification employs repetitive cycles of duplication such as using the “polymerase chain reaction”, the polymerase may be, e.g., a heat stable polymerase, e.g., of *Thermus aquaticus* or other such polymerases, whether heat stable or not.

“Contacting” in the context of target selection means incubating a oligonucleotide library with 15 target molecules. “Target molecule” means any molecule to which specific aptamer selection is desired. “Essentially homologous” means containing at least either the identified sequence or the identified sequence with one nucleotide substitution. “Isolating” in the context of target selection means separation of oligonucleotide/target complexes, preferably DNA/protein complexes, under conditions in which weak binding oligonucleotides are eliminated.

20 By “split synthesis” it is meant that each unique member of the combinatorial library is attached to a separate support bead on a two column DNA synthesizer, a different thiophosphoramidite or phosphoramidite is first added onto both identical supports (at the appropriate sequence position) on each column. After the normal cycle of oxidation (or sulfurization) and blocking (which introduces the phosphate, monothiophosphate or dithiophosphate linkage at this position), the 25 support beads are removed from the columns, mixed together and the mixture reintroduced into both columns. Synthesis may proceed with further iterations of mixing or with distinct nucleotide addition.

Aptamers may be defined as nucleic acid molecules that have been selected from random or 30 unmodified oligonucleotides (“ODN”) libraries by their ability to bind to specific targets or “ligands.” An iterative process of *in vitro* selection may be used to enrich the library for species with high affinity to the target. The iterative process involves repetitive cycles of incubation of the library with a desired target, separation of free oligonucleotides from those bound to the

target and amplification of the bound ODN subset using the polymerase chain reaction (“PCR”). The penultimate result is a sub-population of sequences having high affinity for the target. The sub-population may then be subcloned to sample and preserve the selected DNA sequences. These “lead compounds” are studied in further detail to elucidate the mechanism of interaction with the target.

The present inventors recognized that it is not possible to simply replace thiophosphates in a sequence that was selected for binding with a normal phosphate ester backbone oligonucleotide. Simple substitution was not practicable because the thiophosphates can significantly decrease (or increase) the specificity and/or affinity of the selected ligand for the target. It was also recognized that thiosubstitution leads to a dramatic change in the structure of the aptamer and hence alters its overall binding affinity. The sequences that were thioselected according to the present methodology, using as examples of DNA binding proteins both NF-IL6 and NF- $\kappa$ B, were different from those obtained by normal phosphate ester combinatorial selection.

The present invention takes advantage of the “stickiness” of thio- and dithio-phosphate ODN agents to enhance the affinity and specificity to a target molecule. In a significant improvement over existing technology, the method of selection concurrently controls and optimizes the total number of thiolated phosphates to decrease non-specific binding to non-target proteins and to enhance only the specific favorable interactions with the target. The present invention permits control over phosphates that are to be thio-substituted in a specific DNA sequence, thereby permitting the selective development of aptamers that have the combined attributes of affinity, specificity and nuclease resistance.

In one embodiment of the present invention, a method of post-selection aptamer modification is provided in which the therapeutic potential of the aptamer is improved by selective substitution of modified nucleotides into the aptamer oligonucleotide sequence. An isolated and purified target binding aptamer is identified and the nucleotide base sequence determined. Modified achiral nucleotides are substituted for one or more selected nucleotides in the sequence. In one embodiment, the substitution is obtained by chemical synthesis using dithiophosphate nucleotides. The resulting aptamers have the same nucleotide base sequence as the original aptamer but, by virtue of the inclusion of modified nucleotides into selected locations in the sequences, improved nuclease resistance and affinity is obtained.

## EXAMPLE 1

S-ODN, S<sub>2</sub>-ODN and monothio-RNA Split and Pool Synthesis

A split and pool synthesis combinatorial chemistry method was developed for creating combinatorial S-ODN, S<sub>2</sub>-ODN and monothio-RNA libraries (and readily extended to 5 unmodified ODNs-whether single strand or duplex). In this procedure each unique member of the combinatorial library was attached to a separate support bead. Targets that bind tightly to only a few of the potentially millions of different support beads can be selected by binding the targets to the beads and then identifying which beads have bound target by staining and imaging techniques. The methodology of the present invention allowed the rapid screening and 10 identification of aptamers that bind to proteins such as NF-κB using a novel PCR-based identification tag of the selected bead.

The dA, dG, dC and dT phosphoramidites were purchased from Applied Biosystems (Palo Alto, CA) or Glen Research (Sterling, VA). The Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide) was from Glen Research. The Taq polymerase kits were from Applied Biosystems. 15 The TA Cloning kit was from Invitrogen. The Klenow DNA polymerase I was from Promega. Polystyrene beads (60-70 μm) with non-cleavable hexaethyleneglycol linkers with a loading of 36 μmol/g were from ChemGenes Corp (Ashland, MA). The Alexa Fluor 488 dye was from Molecular Probes, Inc (Eugene, OR). The dA, dG, dC and dT thiophosphoramidites were synthesized as previously described ( Yang, X-B., Fennewald, S., Luxon, B.A., Aronson, J., 20 Herzog, N. and Gorenstein, D.G., "Aptamers containing thymidine 3'-O-phosphorodithioates: Synthesis and binding to Nuclear Factor-κB, *J. Bioorganic and Medicinal Chemistry*, 9, 3357-3362 (1999) and refs therein). The ODNs and S-ODNs used in the study were synthesized on a 1-μmol scale on an Expedite 8909 System (Applied Biosystems) DNA Synthesizer.

Synthesis of S-ODN and S<sub>2</sub>-ODN libraries. Standard phosphoramidite and thiophosphoramidite 25 chemistry was used for the S-ODN and S<sub>2</sub>-ODN libraries, respectively. The libraries were prepared on a 1 μmole scale of polystyrene beads. The downstream and upstream primers, 5'-GGATCCGGTGGTCTG-3' (SEQ ID NO:1) and 5'-CCTACTCGCGAATTC-3' (SEQ ID NO:2) were synthesized in parallel on a two-column DNA synthesizer (Expedite 8909, Applied Biosystems). Following the 5'-primer, the sequences programmed on the synthesizer for the 30 combinatorial S-ODN library were 5'-\*CA\*GT\*TG\*AG\*GG\*GA\*CT\*TT\*CC\*CA\*GG\*C-3' (SEQ ID NO:3) on column 1 and 5'-\*cC\*tG\*cA\*cA\*tC\*tC\*aG\*gA\*tG\*aC\*tT\*t-3' (SEQ ID NO:4) on column 2. The sequences programmed for the combinatorial S<sub>2</sub>-ODN library were 5'-

ATGT\*AGCC\* A\*GCTAGT\*CTG\*TCAG-3' (SEQ ID NO:5) on column 1 and 5'-CGCC\*cAGT\*g\*aAG GTG\*gaA\*CCCC-3' (SEQ ID NO:6) on column 2. The 3'-primer sequence completed the 52-mer programmed on the synthesizer.

A "split and pool" occurred at each position indicated by an asterisk in order to synthesize the 5 combinatorial region for the S-ODN and S<sub>2</sub>-ODN. The lower case letter indicates a 3'-thioate linkage, the upper case letter indicates a 3'-phosphate linkage, while the lower case bold letter indicates a 3'-dithioate linkage. The coupling yield was typically upwards of 99% as determined by the dimethoxytrityl cation assay. Sulfurization chemistry used the Beaucage reagent. The 10 fully protected S-ODN or S<sub>2</sub>-ODN combinatorial libraries with the non-cleavable linker beads were treated with concentrated ammonia at 37 °C for 21 hours to remove the protecting groups while allowing the ODN to remain attached to the beads. The S-ODN or S<sub>2</sub>-ODN bead-based 15 single-strand (ss) DNA library was washed with double distilled water. The ssDNA library (typically 1-3 mg of support beads) was converted to a double-strand (ds) DNA by Klenow DNA polymerase I reaction in the presence of DNA polymerase buffer, dNTP mixture and reverse primer according to the manufacture. The dsDNA library was washed twice with phosphate-buffered saline (PBS).

Figure 1 is a flow diagram of one example for the solid-phase synthesis of a one-bead one-S/S<sub>2</sub>-ODN library. In the first cycle, in column 1, a phosphoramidite dC was used to form a dinucleotide phosphotriester dGC *via* a phosphotriester linkage, in column 2, a phosphoramidite 20 T was used to form a dinucleotide thiophosphotriester dGt *via* a phosphothiotriester linkage. Upon pooling, the end products are a mixture of two kinds of bead-bound dinucleotides include phosphorotriester and phosphothiotriester. After splitting and pooling through three such cycles the eight (2<sup>3</sup>) possible ODN and/or S-ODN tetraoligonucleotides are represented on separate beads. A lowercase letter denotes a 3'-thioate, while an uppercase letter denotes a 3'-phosphate. 25 The S<sub>2</sub>-ODN library was generated by replacing the phosphoramidite with a thiophosphoramidite globally in column 2. The sulfurization step immediately followed the thiophosphoramidite coupling step.

Labeling NF-κB p50/50 protein with Alexa Fluor 488. To 0.5 ml of p50/50 protein (0.215 mg/ml, expressed and purified (King, D., et al. (2002) Combinatorial selection and binding of 30 phosphorothioate aptamers targeting human NF-κB RelA (p65) and p50. *Biochemistry*, 41, 9696 – 9706, relevant portions incorporated herein by reference) in PBS containing 30% glycerol was added 50 μl of 1 M bicarbonate. The protein was transferred to a vial of reactive Alexa Fluor

488 dye and stirred at room temperature for 1 hr. Fluorescently labeled protein was purified according to procedures from Molecule Probes, Inc. The labeled protein was stored at 4 °C in the dark.

Alexa Fluor 488 labeled NF-κB p50/p50 binding to beads, selection of beads. A portion of the 5 ds S-ODN or S<sub>2</sub>-ODN library (ca. 3.0 mg of the beads) was suspended in 300 μl of blocking buffer (PBS containing 0.05% Tween-20) and incubated at room temperature for 1 hr in a microcentrifuge tube. The beads were washed with 300 μl of PBS and pelleted by centrifugation. The beads were suspended in 300 μl of Alexa Fluor 488 labeled NF-κB p50/p50 (0.07 μg/μl) at room temperature for two hrs and then washed with blocking buffer (2 x 300 μl) 10 and PBS (2 x 300 μl). A portion of the beads were transferred to a slide and viewed under fluorescent microscope. Individual beads with the highest fluorescence intensity were removed by a micropipette attached to a micromanipulator, sorted into PCR microcentrifuge tubes and washed with 8M urea (pH 7.2) to remove the bound protein.

One-bead one-PCR amplification and sequencing of PCR product. A selected single bead was 15 mixed with the following PCR components: 6 μl of 25 mM MgCl<sub>2</sub> (8 μl for 15, 10 and 8mer primers), 0.5 μl of Taq polymerase (5 units/μl), 1 μl of 8 mM dNTP, and 10 μl of PCR buffer and 1 μl of 40 mM primers. The PCR was run on a GeneAmp PCR system 2400 (Perkin Elmer). The PCR reaction mixtures were thermal cycled using the following scheme for amplification: 94 °C for 5 min (1 cycle); 94 °C for 2 min, 55 °C for 2 min (35 °C for 10 and 8mer primers), 72 20 °C for 2 min (35 cycles); and 72°C for 7 min (1 cycle). The PCR products were analyzed on a 15% native polyacrylamide gel. The PCR product was cloned using the TA Cloning procedure (Invitrogen) and sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

One-bead one-oligonucleotide libraries. A primary consideration for designing a one-bead, one-ODN library using phosphoramidite chemistry was defining suitable bead linker chemistry 25 where the ODNs can be synthesized and yet remain attached covalently to the beads after full deprotection. Additional considerations include development of the split synthesis method for construction of the ODN library, screening bead-based ODN libraries in aqueous media for one-bead binding assays and sequencing of the ODN bound on the individual bead. Although long-chain alkylamine controlled-pore glass (LCA-CPG) (Pierce Chemical Co., Rockport, IL) has been 30 used for many years for efficient ODN synthesis, LCA-CPG may not always be suitable for generation of one-bead, one-ODN libraries. The size, homogeneity and the swelling of CPG are factors to consider when selecting a chemistry for a one-bead, one-ODN library. For example,

one disadvantage of the CPG linker chemistry available currently is that ODNs are cleaved from the solid support during the ammonia deprotection step. An advance in solid support chemistry has been the ability to synthesize ODNs on more uniform polystyrene beads. Importantly, using chemistry with a non-cleavable hexaethyleneglycol linker attaching the first phosphoramidite

5 (ChemGenes Corp.), the synthesized ODNs are still attached covalently to the beads after full base and phosphate ester deprotection. In this procedure each unique ODN chemical entity in the combinatorial library is attached to a separate support bead. Selection of a bead-based ODN combinatorial library can then be carried out by binding the bead library of ODNs to a target protein under high stringency conditions where only a few beads show binding.

10 Following the *in vitro* combinatorial selection method for identification of selected ODN sequences, the ODN sequence on the selected beads may be identified. For example, 5' and 3' fixed ODN primer sequences flanking the combinatorial library segment of the ODN may be used to aid in the identification. Fixed primer regions allow PCR amplification of the sequence as well as Klenow extension of the ssDNA attached covalently to produce a combinatorial  
15 library of dsODN attached to the beads.

Primers design for one-bead one-ODN. Initially, a template ODN with a predetermined 14mer sequence region flanked by two 18mer primers on the beads were synthesized (ODN 1 in Table 1). Its ability to support one-bead one-PCR amplification was studied for several individual beads. The PCR product was cloned using the TA Cloning procedure and sequenced on an ABI  
20 Prism 310 Genetic Analyzer. The desired sequence was confirmed. Although 18mer or longer primers are generally used in PCR amplification, shorter primers are attractive since the size of the ODN is limited by the synthesis yields for long ODNs.

**Table 1.** ODNs on beads and primers

ODNs on beads used as templates

25 ODN1:5'-ATGCCTACTCGCGAATTC-CCAGGAGATTCCAC-GGATCCGGTGGTCTGTTC-  
Bead (SEQ ID NO:7)

ODN2:5'-CCTACTCGCGAATTC-AGTTGAGGGGACTTCCCAGGC-  
GGATCCGGTGGTCTG-Bead (SEQ ID NO:8)

Primers

30 Upstream primers (SEQ ID NO:9) Downstream primers (SEQ ID NO:10)

18mer :	5'-ATGCCTACTCGCGAATT-3'	5'-GAACAGACCACCGGATCC-3'
15mer :	5'-CCTACTCGCGAATT-3'	5'-CAGACCACCGGATCC-3'
10mer :	5'-CCTACTCGCG-3'	5'-CAGACCACCG-3'
8mer :	5'-CCTACTCG-3'	5'-CAGACCAC-3'

5 Note : the 15-mer, 10-mer and 8-mers are nested oligos of: SEQ ID NO:9 and SEQ ID NO:10, respectively.

Consequently, longer combinatorial sequence libraries would be possible with shorter primer sequences; in addition, shorter primer sections will reduce non-specific binding of target proteins 10 to the ODN bead library. To study the primer length requirement for one-bead one-PCR amplification, a series of primers with varying lengths (8mer, 10mer and 15mer; see Table 1) were designed and synthesized to hybridize to the 52mer template ODN containing both 5' and 3' primer regions (ODN 2 in Table 1) on the support beads. The PCR products of these primers were monitored by 15% polyacrylamide gel electrophoresis. The PCR conditions were 15 optimized for each pair of primers of varying length. No detectable band was observed with the 8mer primers, even at the highest concentration tested (data not shown). A weak band was detected with the 10mer primers, while a strong band was observed with 15mer primers (data not shown). The fidelity in the Taq polymerase amplification yielding the ODN products was confirmed by cloning and sequencing (data not shown). These results suggest that ODNs with 20 primer lengths of 10 nt or greater are required for efficient PCR amplification. In this study, 15 mer primers were selected for the following studies.

Generation of a self-encoded S/S<sub>2</sub>-ODN library. Figure 1 shows the synthetic scheme for the introduction of many copies of a single, chemically pure S-ODN or S<sub>2</sub>-ODN onto each bead, a "mix and separate" split synthesis method was used. A two-column DNA synthesizer was used 25 for constructing the library. The normal phosphate backbone linkages were generated using standard phosphoramidite monomers *via* oxidation in column 1, while the phosphorothioate or phosphorodithioate linkages were synthesized using standard phosphoramidite or thiophosphoramidite monomers *via* sulfurization in column 2, respectively. Two sequences of the same length are programmed for each column and are designed such that the bases are 30 different at every equal position not only for diversifying base compositions but also for coding a phosphate, phosphorothioate or phosphorodithioate linkage. Thus, on an Expedite 8909 DNA synthesizer with dual columns, for example, onto column 1 a phosphoramidite (for example: dC) is coupled to the bead and after completion of oxidation, the resulting product is nucleotide (dC)

with a phosphotriester linkage. On column 2, a nucleoside phosphorothioate or phosphorodithioate is introduced with a different base (dT for example). The support beads from the two columns are mixed and resplit and in the second cycle, additional phosphoramidites or thiophosphoramidites are introduced, followed by oxidation and sulfurization reactions 5 individually in columns 1 and 2.

After additional coupling steps and after final split/pool synthesis is completed, the end products comprise a combinatorial library of ODNs with varying thioate/dithioate or normal phosphate ester linkages at varying positions along the ODN strand attached to the support. Each bead 10 contains a single chemical entity with a specified backbone modification that is identified by the base. In the above example, any dC at position 1 of the sequence will be a 3'-phosphate while a dT at position 1 would indicate that it contains a 3'-thiophosphate. This scheme was applied to synthesize a library of 4096 ( $2^{12}$ ) one-bead, one-S-ODN. This library included a 22-nucleotide 15 combinatorial sequence (12 split/pool steps) flanked by 15 nucleotide defined primer regions at the 5' and 3' ends (see Table 1). The 3' ends of the sequences were attached to the polystyrene beads. As noted above, the defined primer sequences were incorporated to allow PCR 20 amplification and identification of the ODN sequence on the selected beads. Thus, the downstream primers were first automatically synthesized in parallel on the two columns. The S-ODN sequences of the combinatorial 22-mer segment on each column were programmed for each column and were generated by introducing a phosphorothioate linkage on every other base 25 in column 2, following the "split and pool" approach. The identical upstream primer sequences were then completed on both columns. As described below, a smaller S<sub>2</sub>-ODN library was created in similar fashion.

Selection and sequencing of the S-ODN beads. Binding of the transcription factor NF- $\kappa$ B p50/p50 homodimer and selection of specific beads was demonstrated by first converting the 25 single-stranded S-ODN to dsDNA since the NF- $\kappa$ B transcription factor binds to DNA duplexes. The single-stranded 52-mer S-ODN combinatorial library (typically 1-3 mg of beads) was converted to dsDNA by Klenow DNA polymerase I reaction in the presence of DNA polymerase buffer, dNTP mixture and reverse primer. Therefore, one strand of the duplex potentially contained thiophosphate backbone substitutions in the combinatorial library segment and the 30 other complementary strand included an unmodified phosphate backbone ODN. A duplex DNA library in which both strands contain S-ODN modifications could also be generated using a Klenow reaction with no more than three dNTP( $\alpha$ S). Because the S-ODN strand attached to the support was chemically synthesized using phosphoramidite chemistry, each thiophosphate is a

mixture of R<sub>P</sub> and S<sub>P</sub> stereoisomers. The beads were suspended in a diluted solution of NF-κB p50/p50 homodimer labeled with the Alexa Fluor 488 dye at room temperature for 2 hrs.

Figures 2A – 2C show a portion of the beads when viewed under both light and fluorescence microscope, after washing with PBS containing 0.1% Tween 20 and PBS to minimize nonspecific binding. Typically, a few positive beads were intensely stained when viewed by fluorescence, while the majority of the beads remained unstained as shown in Fig. 2B. With the aid of a micropipette coupled to a micromanipulator, the intensely stained beads were retrieved as shown in Fig 2C.

Figure 2A is an aliquot of S-ODN beads bound to NF-κB p50/p50 homodimer protein labeled with the Alexa Fluor 488 dye viewed under light microscopy. Figure 2B shows the same beads viewed under fluorescence microscopy, in which a positive green bead stained with Alexa Fluor 488 dye can be easily identified in a background of many hundreds of nonreactive beads. Finally, Figure 2C shows that a single positive bead can easily be retrieved with a micromanipulator under fluorescence microscope.

Five positive beads from the S-ODN library were selected. Each individual bead was washed thoroughly with urea to remove the protein and was directly used for the “one-bead, one-PCR” amplification using the 5’ and 3’ end primers described above. The PCR product was cloned and sequenced. Table 2 lists four of the S-ODN sequences obtained.

**Table 2.** S-ODNs/S<sub>2</sub>-ODNs sequences identified from the screen with NF-κB p50/p50 protein<sup>a</sup>  
20 S-ODN selection

	Automated sequence	Deduced S/S <sub>2</sub> -ODN sequence
	CTGTGAGTCGACTGATGACGGT	CtGTGAGtCGACTgAtGaCGGt
	AGTTGAGTCGAAGGACCCATT	AGTTGAGtCGAaGgACCCAtTt
	CGTCAAGTCTCAGTTCCCATT	CGTcAAGtCtCaGTTCCCAtTt
25	AGTCAAGTCGAAGTTCCACGGT	AGTcAAGtCGAaGTTCCaCGGt
	(SEQ ID NOS:11-14)	(SEQ ID NOS:15-18)

**S<sub>2</sub>-ODN selection**  
ATGTAGCCAGCTAGTCTGTCAG  
CGCCAGCCAAAGGTGCTGTCAG

CGCCCAGTGGCTAGTGAACCCC	CGCCcAGTgGCTAGTgaACCCC
ATGTAGCCGAAGGTGGAACCCC	ATGTAGCCgaAGGTGgaACCCC
CGCCAGCCGAAGGTGGAACCCC	CGCCAGCCgaAGGTGgaACCCC

(SEQ ID NOS:19-23) (SEQ ID NOS:24-28)

5

<sup>a</sup>The lower case letter indicates a 3'-thioate linkage. The lower case bold letter indicates a 3'-dithioate linkage. <sup>b</sup>No 3'-dithioate linkages are present in this strand.

Binding and Selection of combinatorial library of S<sub>2</sub>-ODN beads. S<sub>2</sub>-ODNs generally bind even  
10 more tightly to proteins than unsubstituted or S-ODN analogues. Thus, it is significant that this  
S-ODN bead-based combinatorial selection method may be applied to dithiophosphate backbone  
substitutions, since *in vitro* combinatorial selection is only possible for thiophosphate substituted  
ODNs with limited P-chirality. To demonstrate how the present invention may overcome this  
15 limitation, a small one-bead, one-S<sub>2</sub>-ODN library was synthesized consisting of a pool of 32 (2<sup>5</sup>)  
sequences to allow further optimization of *in vitro* or bead-based S-ODN selected sequences.  
Chemical synthesis of S<sub>2</sub>-ODN avoids problems created by a mixture of diastereoisomers of  
20 chemically synthesized S-ODN. The random region (5'-CGCCcAGTgaAGGTGgaACCCC-  
3')(SEQ ID NO:28) in column 2 was identified as a S-ODN sequence derived from an *in vitro*  
combinatorial selection methodology that binds the NF-κB p50/p50 protein with high affinity  
25 (<20 nM) (All lower case letters indicate enzymatically synthesized chiral 3'-thioate linkages).  
The programmed combinatorial region sequence (5'-ATGTAGCCAGCTAGTCTGTCAG-  
3')(SEQ ID NO:19) in column 1 was designed such that the bases at each 3'-dithioate position  
were different from the bases in column 2 at each equal position further allowing base sequence  
30 to identify backbone substitution. Thiophosphoramidite chemistry with sulfurization was used to  
generate 3'-dithioate linkages. Only the previous 3'-monothioate linkages were replaced with  
3'- dithioate linkages. The “split and pool” step followed most of the dithioate modifications.  
S<sub>2</sub>-ODNs by selecting beads binding fluorescently labeled NF-κB p50/p50 homodimer were also  
identified, followed by PCR amplification of 5 individually selected S<sub>2</sub>-ODN beads and cloning  
and sequencing of the PCR products. The sequences are also listed in Table 2.

30 The nucleic acid “aptamers” previously selected by incubating the target (protein, nucleic acid or  
small molecule) with the combinatorial library were then separated. The bound fractions were  
then amplified using PCR and subsequently reincubated with the target in a second round of  
screening. These iterations are repeated (often 10-20 cycles) until the library is enhanced for  
sequences with high affinity for the target. Aptamers selected from combinatorial RNA and

DNA libraries have generally had normal phosphate ester backbones, and so would generally be unsuitable as drugs or diagnostics agents that are exposed to serum or cell supernatants because of their nuclease susceptibility. Rapid degradation of natural ODNs used as antisense agents or aptamers by nucleases in serum or cells necessitates chemical modification of the ODNs.

5 Among a large variety of modifications, S-ODN and S<sub>2</sub>-ODN render the agents more nuclease resistant. The first antisense therapeutic drug uses a modified S-ODN (CIBA Vision, A Novartis Company). The S<sub>2</sub>-ODNs also show significant promise, however, the effect of substitution of more nuclease-resistant thiophosphates cannot be predicted, since the sulfur substitution can lead to significantly decreased (or increased) binding to a specific protein (Milligan, J.F. and  
10 Uhlenbeck, O.C. (1989) Determination of RNA-protein contacts using thiophosphate substitutions. *Biochemistry*, 28, 2849-2290.; and Yang, X. unpublished results) as well as structural perturbations (Volk, D. *et al.* *Bioorganic Chemistry* 2002, in press) and thus it is not possible to predict the effect of backbone substitution on a combinatorially selected aptamer.  
15 Thus, if at all possible, selection should be carried out simultaneously for phosphate ester backbone substitution as well as the base sequence. Recently, an *in vitro* combinatorial selection of thioaptamers from random or high-sequence-diversity libraries based on their tight binding to the target (e.g. a protein or nucleic acid) of interest was demonstrated by one or more of the present inventors.

Oligonucleotides possessing high fractional substitutions of monothio/dithioate internucleotide linkages appear to be “stickier” towards proteins than normal phosphate esters, and therefore thioaptamers with complete thiophosphate backbone substitutions appear to lose much of their specificity. This increased affinity is partly due to the fact that the thioate groups only poorly coordinate hard cations such as sodium ions, and thus the thioaptamers serve as “bare” anions and don’t require any energy to strip away the neutralizing cations to bind to proteins. This 20 observation of the increased affinity is of great importance to modified-ODN design as proteins recognize DNA at both the bases and phosphate esters. In previous studies, it was demonstrated that binding of S<sub>2</sub>-ODNs to a protein target requires only a limited number of phosphorodithioate linkages in a specific ODN sequence to achieve very high affinities (Gorenstein, D.G., *et al.*, U.S. Patent, 6,423,493, relevant portions incorporated herein by reference).  
25 These results demonstrate that a split and pool synthesis may be used to develop S-ODN, S<sub>2</sub>-ODN and RNA libraries (which may also include unmodified ODNs-whether single strand or duplex). In this procedure each unique member of the combinatorial library was attached to a  
30

separate support bead. Targets that bind tightly to only a few of the potentially millions of different support beads were selected by binding the targets to the beads and then identifying which beads have bound target by staining and imaging techniques. The methodology of the present invention allowed the rapid screening and identification of aptamers that bind to proteins, 5 e.g., NF- $\kappa$ B, using a novel PCR-based identification tag of the selected bead.

These results demonstrate that the methodology can be applied to other backbone or base modifications that are compatible with templates containing these modifications. It is important that not only the S-ODNs but even the S<sub>2</sub>-ODNs are capable of acting as templates recognized by 10 DNA polymerases for PCR amplification of selected S<sub>2</sub>-ODN beads. This demonstrates that nucleic acid analogues with phosphorodithioate linkages can be used as a template in the nucleotidyltransferase reaction catalyzed by DNA polymerases. Likewise, polyamide nucleic acid (PNA) lacking the phosphate backbone may be recognized as a template for the polymerase reaction.

15 *In vitro* selection of combinatorial libraries of S<sub>2</sub>-ODNs is not possible because dNTP ( $\alpha$ S<sub>2</sub>) is not a substrate for polymerases. The split synthesis, bead-based S<sub>2</sub>-ODN library selection method of the present invention is the only method and/or library for identifying both optimal number and location of dithioate substitutions as well as base sequences for these S<sub>2</sub>-ODN aptamers. Additionally, even for the thioate library selection, the *in vitro* methods involving 20 iterative cycles of selection isolation and reamplification of the bound members of the library by PCR amplification are very time consuming. In contrast, the single cycle of split/pool synthesis, selection and identification of the present invention circumvents the need for the iterative cycles of amplification, isolation and reamplification. The split pool bead-based method and library of the present invention allows for the identification of the positions of any 3'-monothioate/dithioate linkages.

25 Although the beads were screened against a target protein labeled with a fluorescent dye, the beads can also be screened directly against the unmodified transcription factor. The binding of the NF- $\kappa$ B to a specific sequence can be detected using a primary anti-NF- $\kappa$ B antibody, followed by a secondary antibody conjugated to a marker molecules including fluorescein or rhodamine for fluorescence microscope (Yang, X. unpublished results).

30 To confirm the selection results, the S-ODN: 5'-CtGTGAGtCGACTgAtGaCGGt-3' (small letters represent location of 3'-thioates), was independently synthesized on the non-cleavable linker bead support, hybridized with its complementary ODN and then mixed again with the NF-

$\kappa$ B p50/p50 protein labeled with the Alexa Fluor 488 dye. The fluorescence intensity of all of the beads viewed under the fluorescence microscope was qualitatively similar to the intensity of the selected bead containing this sequence within the combinatorial library. These results demonstrate that the primer regions do not contribute to the binding of the NF- $\kappa$ B p50/50.

5 Quantitative studies on the affinities of the selected S-ODNs and S<sub>2</sub>-ODN duplexes to the protein along with selection from a large combinatorial library ( $10^6 \sim 10^8$ ) to NF- $\kappa$ B are in progress.

In earlier studies a thioaptamer clone obtained from an *in vitro* combinatorial selection experiment (15 rounds of selection) bound to NF- $\kappa$ B p50/p50 with an apparent dissociation constant < 5 nM (thiophosphate modification 5' to each dA residue: 5'-

10 GGGGTTCCACCTTCACTGGGCG-3'•3'-CCCAAGGTGGAAGTGACCCGC-5')(SEQ ID NOS:29 & 30). A chemically synthesized thioaptamer of the same sequence bound with a dissociation constant of < 20 nM. It should be noted, however, that each chemically synthesized thioaptamer consists of a diasteromeric mixture containing  $2^n$  different stereoisomers, where n is the number of thiophosphates ( $2^7 = 128$  for the NF- $\kappa$ B p50/p50 selected thioaptamer). To 15 determine the importance of the thiophosphate substitutions in the thioaptamer toward the NF- $\kappa$ B p50/p50 homodimer, a tight binding 15th round thioaptamer clone was synthesized by PCR with a nucleotide mix containing dATP instead of dATP( $\alpha$ S), and showed no binding of the normal phosphoryl backbone aptamer to NF-  $\kappa$ B p50/p50 protein, supporting the critical role played by the thiophosphates.

20 Phosphoramidite chemistry has been widely used for the synthesis of S-ODNs because of its automation, high coupling efficiency and ease of site-specific thioate linkage incorporation. Synthesis of S-ODNs may be carried out by, e.g., forming an internucleoside phosphite linkage followed by sulfurization of the phosphite triester to a phosphorothioate. The resulting S-ODNs are a mixture of diastereoisomers, and consequently the diasteromeric S-ODN mixtures may 25 have variable biochemical, biophysical and biological properties. Each bead then contains a library of monothioate aptamers (a library of libraries) since each bead contains the identical sequence and position of thiophosphate substitution, but represents a mixture of diastereomers introduced through the new monothiophosphate chiral centers. Stereocontrolled synthesis of a stereodefined S-ODN library may also be used to determine which is the best aptamer that binds 30 to the protein, or may even be used to select a thioaptamer (or thioaptamer library) that has high affinity for the target protein or biomolecule. The binding data indicated that diastereoisomeric mixture libraries have good selectivity and affinity, although not as high as pure stereoisomers.

Another possible solution lies in the synthesis of modifications that are achiral at phosphorus, such as the above S<sub>2</sub>-ODN thioaptamer library study. In addition dithioates appear to have greater "stickiness" to proteins than the thioates or unmodified ODN backbone.

The present invention may also be used to identify different nucleotide sequence(s) and/or to  
5 identify the backbone modification. S-ODN and S<sub>2</sub>-ODN libraries were also created that differ only in the position of phosphate or dithioate but not in its base sequence. It is known that positions of thiophosphates in a mixed backbone S-ODN sequence can be determined by reaction of the S-ODN with iodoethanol followed by base catalyzed cleavage of the thiophosphate triester. The feasibility of this approach for identifying location of thioate  
10 linkages has been demonstrated by the present inventors, and is often independent of base sequence.

The search for other split synthesis, bead-based combinatorial libraries containing base modifications and hybrid backbones with phosphate ester, thioates, dithioates or potentially neutral methylphosphonates or even peptide nucleic acid chimeras with improved properties,  
15 such as enhanced binding-affinity to a specific protein, increased biological stability, and improved cellular uptake, may be achieved by the split synthesis combinatorial selection method described here.

By the split/pool method with two columns 2<sup>N</sup> different members of the library for N split/pool steps have been created. More columns (M) may also be used with the present invention to  
20 permit synthesis of M<sup>N</sup> different beads with one unique thioaptamer sequence on each bead. The limit to the size of the combinatory library is the number of steps (N) and the number of columns (M) and of course the total number of beads, which generally is in the range of 10<sup>6</sup> or more depending upon the size of the beads and synthesizer columns. Recently, aptamer beads on 15-  
25 20  $\mu$ m beads was achieved (Yang, unpublished) and thus a 40-fold increase in the library size is possible. These results demonstrate that small bead sizes may be used effectively to produce more complex libraries at reduced cost and making more efficient use of reagents. The use of 15-20  $\mu$ m beads also demonstrates the scalability of the present invention. Finally, these results demonstrate that library sizes comparable to those created by *in vitro* combinatorial selection methods by using mixtures of phosphoramidites/thiophosphoramidites (up to 8 different species)  
30 at selected positions in a given synthesis step may be created. The methodology of the invention may even be used to create a library of libraries of beads, each bead containing a library of any complexity. Using the present invention a screener may easily create 10<sup>6</sup> beads with 10<sup>8</sup>

combinatorial library members on each bead – total diversity in principle is thus  $10^{14}$ , the same as in *in vitro* combinatorial selection libraries.

Sulfur substitution in aptamers alters the binding affinity and sequence that is obtained by *in vitro* combinatorial selection methods. Post-selection phosphorothioate modifications of *in vitro*

5 combinatorially selected sequences can thus result in thioaptamers in which affinity cannot be reliably predicted. The simultaneous selection for both avoids this difficulty. The bead-based split synthesis, selection and PCR identification of combinatorial aptamer libraries now provides a means to combinatorially select both monothioate and dithioate variations on aptamers.

Flow cytometry sorting of thioaptamer bead-based library. The present inventors also

10 demonstrated the successful application of high throughput/multi-color flow cytometry and bead sorting to screen aptamer bead libraries for those beads which bind to, e.g., a target protein. Modifications may be made to the flow cytometer to make it more amenable to bead identification and isolation. For example, bead fluorescence and forward scatter were the two parameters chosen for real-time characterization of each aptamer bead passing the first sort point  
15 of a custom-built flow cytometer/sorter. Other scanning and sorting parameters may be used to select, isolate, view, designate, characterize, etc. the beads through a flow cytometer as will be apparent to those of skill in the art of cytometric analysis.

In operation, “positive” beads (contain thioaptamer-bound target protein, the target protein was fluorescent-labelled with Alexa 488 dye) were easily sorted from negative beads. Flow

20 cytometry may be used to replace, e.g., visual fluorescence microscope identification of beads containing bound target protein and the need to isolate the individual “positive” beads with a micromanipulator. The flow-sorted “positive” beads can then be subjected to, e.g., one-bead PCR to identify the thioaptamer that binds the target protein. The sorted “positive” beads may also be subjected to SELDI-MS analysis to confirm the identity of the bound protein (via  
25 molecular ion characterization). In cases where the “positive” bead’s thioaptamer might have bound not only the target protein but other proteins in a sample, e.g., a secondary or even tertiary, etc. protein, SELDI-MS may be used to identify this event through the detection of multiple molecular ions. These results demonstrate that fluorescently protein-labeled beads are detected and differentiated from one another in a flow system in order to, e.g., sort out certain  
30 portions of the beads.

As shown in Figures 3A-3D, and as a demonstration of the use of the one-bead, one-ODN:protein system using dual color sorting. The dsDNA  $\kappa$ B consensus sequences in the Ig $\kappa$

gene were immobilized onto 15-20 micron polystyrene microspheres. The DNA bound beads were then incubated with purified NF- $\kappa$ B p50 and p65 proteins, respectively. DNA transcription factor complexes were detected with primary antibodies specific for the NF- $\kappa$ B p50 and p65 proteins followed by an additional incubation with Alexa 488- conjugated secondary antibody for NF- $\kappa$ B p50 and PE- conjugated secondary antibody for p65. The beads were viewed by 5 fluorescent microscopy and then analyzed on the MCU's HiReCS system.

In Figure 3A, a Control Fluorescent Cell Sort (CONTROL.FCS) shows the autofluorescent microspheres in the negative control sample where the beads were unbound. Figure 3 B shows the NF- $\kappa$ B p50 positive beads only, while Figure 3C shows the p65 positive beads only. Finally, 10 Figure 3D shows a mixed bead population. The majority of the "debris" population are the 0.8 micron carrier beads that were used to bring up the volume of the samples since the beads were at a very low dilution. Table 3 shows the population statistics.

Table 3  
Population Statistics

Sample	Total	Region	%Gate
<i>Figure 3A: CONTROL.FCS</i>			
R1: Autofluorescent Beads	10000	9530	95.3
<i>Figure 3B. FCS</i>			
R2: p50 Alexa 488 Positive Beads	10000	35	0.35
<i>Figure 3C. FCS</i>			
R3: p65 PE Positive Beads	20000	3488	17.44
<i>Figure 3D. FCS</i>			
R1: Autofl. Beads & Carrier Beads	1000000	963321	96.33
R2: p50 Alexa 488 Positive Beads	1000000	354	0.04
R3: p65 PE Positive Beads	1000000	935	0.09

15

## EXAMPLE II

### ELISA based Thioaptamer selection-Indirect ELDIA

Although the beads were screened against a target protein labeled with a fluorescent dye, the beads can also been screened directly against the transcription factor. The binding of the NF- $\kappa$ B 20 to a specific sequence can be detected using a primary anti-NF- $\kappa$ B antibody (Rabbit IgG antibody, Santa Cruz Biotechnology, Inc.) followed by a secondary antibody conjugated with Alexa Fluor 488 (goat anti-rabbit IgG from Molecular Probes). Next, several beads were selected for sequencing. The sequencing result were as follows:

E008 Selected sequences

25 5'-CGCCAGCCG<sup>a</sup>AGGTGCTGTCAG-3' (SEQ ID NO:31)

5'-ATGTAGCCA<sup>a</sup>AGGTG<sup>ga</sup>ACCCC -3' (SEQ ID NO:32)

5'-CGCCcAGTgaAGGTGCTGTCAG -3' (SEQ ID NO:33)  
5'-CGCCcAGTAGCTAGTCTGTCAG -3' (SEQ ID NO:34)

It was observed that the phosphorodithioate linkage (s) in the selected above sequences were  
5 different from those of the screening against the fluorescently labeled NF- $\kappa$ B p50. This result  
suggests that some of the binding sites of NF- $\kappa$ B p50 protein may be preoccupied by fluorescent  
molecules.

### EXAMPLE III

#### Labeling of the ODN with fluorescent dyes

10 When synthesizing combinatorial libraries or specific thioaptamer sequences on beads, one may  
also identify beads by attaching 2 or more fluorescent dyes to the ODN either at the 5' or 3' ends  
or internally by using phosphoramidites with specific fluorophors attached. By using 1- 3 (or  
more) fluorophors at 2-3 or more different levels (individual nucleosides), it is possible to  
identify dozens or more of the sequences or libraries by multicolor flow cytometry. (Each bead  
15 can thus be identified by dye A, B and/or C at levels high, medium, low in various combinations:  
thus bead with A(hi), B(medium) and C(low) would be one of dozens of different possible  
combinations.)

Thus it is possible to multiplex using flow cytometry or by randomly placing beads onto, e.g., the  
Texas tongue with hundreds or thousands or more of different microwell holders, random  
20 assortment of thioaptamer beads specific for binding different analytes. Alternatively, it is  
possible to label fluorescently cell extracts with another dye and then bind the protein(s) to the  
beads in conjunction with multicolor flow or surface fluorescence, multiplex diagnostics chip or  
beads, as described hereinbelow.

### EXAMPLE IV

25 Fluorescent tagging of proteins only with SELDI MS to identify proteins expressed differentially

The thioaptamer combinatorial library may be used in conjunction with fluorescent tagging of  
proteins only and SELDI MS to identify proteins differentially expressed in control vs.  
experiment. In this simple two-color assay, a combinatorial library (or a combinatorial library of  
30 libraries) of beads is synthesized, each bead with a single thioaptamer sequence (or a  
combinatorial library of thioaptamer sequences on each bead). In this was we could create up to  
 $10^8$  beads with a single thioaptamer sequence on each bead.

Cell extracts of a sample are labeled fluorescently with a dye (cy3 for example) and a control cell extract is labeled fluorescently with another dye (cy5 for example). Both cell extracts are mixed together and allowed to bind to the bead thioaptamer library. Next, two (2) color flow cytometry is used to compare cy3/cy5 color levels of each bead. If cy3/cy5 level differs from 1, then the  
5 bead may be captured. To determine which protein(s) have been bound to selected thioaptamer bead protein determination technique, e.g., SELDI MS may be used to characterize the bound target further. SELDI MS may be used to determine which proteins have been bound to selected combinatory thioaptamer libraries and also used with single bead PCR to identify which bead(s) in the combinatorial library have bound to protein(s). Pure thioaptamer beads may be placed or  
10 spotted onto a chip or used in conjunction with, e.g., flow cytometry methods to bind the protein expressed differentially in a sample relative to control.

#### EXAMPLE V

##### Fluorescent tagging and SELDI to identify proteins expressed differentially

The thioaptamer combinatorial library may be used in conjunction with fluorescent tagging and  
15 SELDI to identify proteins differentially expressed in control vs. experiment. Combinatorial libraries of thioaptamer sequences on individual beads can be synthesized (for example at position N1 on a split synthesis column 1 use 33% of A, G and T while on column 2 use C thiophosphoramidite to introduce either normal mixture of A/G/T phosphates or C dithiophosphate which can be identified by sequencing the N1 position. In this fashion we could  
20 create up to  $10^8$  beads with  $10^{12}$  combinatorial library members on each bead – total diversity in principle is  $10^{20}$  (of course the actually diversity is only about  $10^{14}$  at best).

Alternatively, the user may create only 100 different split synthesis beads each with up to  $10^{12}$  combinatorial library members on the beads. By using, e.g., 3 or more fluorophors, attached at various levels via phosphoramidite chemistry to the ODN it is possible to use flow cytometry to  
25 identify each bead library. For example, sample cell extracts may be labelled fluorescently with one dye (cy3 for example) and control cell extracts may be labelled fluorescently with another dye (cy5 for example). Use of five (5) color flow cytometry may also be used to compare cy3/cy5 color levels of each bead library that is identified by covalently attached fluorophors to one or more of the thioaptamer(s). If cy3/cy5 level differs from 1, then sort the beads by the  
30 fluorophore tags for each non-unitary cy3/cy5 levels. As with the previous Example, SELDI MS may be used to determine which proteins have been bound to selected combinatory thioaptamer

libraries and also used with single bead PCR to identify which bead(s) in the combinatorial library have bound to protein(s).

In one alternative embodiment of the present invention it is possible to incorporate a modified nt phosphoramidite (at the C-5 pyrimidine position for example) in the combinatorial library

5 sequence positions to create a tag for the libraries and thus create 100 libraries in one split/pool synthesis.

Alternatively, it is possible to use photoactivated crosslinkers to attach the protein to the thioaptamer (e.g., BrU on a single strand). Proteolysis of protein(s) may be used in conjunction with MS to identify the bound peptides and/or proteins. It is also possible, as described

10 hereinabove, to use single-bead PCR to identify which bead(s) from the combinatorial library have bound to the protein(s). Since the BrU is on only 1 strand of the thioaptamer, the other can be sequenced by PCR. To identify the exact thioaptamer sequence that bound to the protein,

then a four (4) column split/pool synthesizer may be needed. Alternatively, it is possible to spot pure thioaptamer or library onto chip and use this spot to bind the differentially expressed protein

15 under the sample relative to control.

#### EXAMPLE VI

##### Synthesized a monothio RNA library

The present inventors have also successfully synthesized a monothio RNA library ( $2^{15}=32768$ ).

Standard phosphoramidite (DNA and RNA) chemistry was used for the monothio RNA library.

20 A 0.5 M 1H-tetrazole in acetonitrile was used as DNA activator. A 0.5 M solution of DCI (dicyanoimidazole) in acetonitrile was used as RNA activator. The libraries were prepared on a

1  $\mu$  mole scale of polystyrene beads (66-70  $\mu$ m). The downstream and upstream primers, 5'-d(GGATCCGGTGGTCTG)-3' (SEQ ID NO:35) and 5'-d(CCTACTCGCGAATTC)-3' (SEQ

25 ID NO:36) were synthesized in parallel on a two-column DNA synthesizer (Expedite 8909, Applied Biosystems).

Following the 5'-primer, the sequences programmed on the synthesizer for the combinatorial mono RNA library were 5'-r(GA\*UC\*CU\*GA\*AA\*CU\*GU\*UU\*UA\*AG\*GU\*UG\*GC\*CG\*AU\*C)-3' (SEQ ID

30 NO:37) on column 1 and 5'-r(cU\*aG\*gA\*cU\*uG\*gC\*aC\*aA\*cC\*gU\*cA\*cA\*cU\*gC\*uA\*u)-3' (SEQ ID NO:38) on column 2. The 3'-primer sequence completed the 61-mer programmed

on the synthesizer.

A "split and pool" occurred at each position indicated by an asterisk in order to synthesize the combinatorial region for the monothio RNA. The lower case letter indicates a 3'-thioate linkage,

the upper case letter indicates a 3'-phosphate linkage. The coupling yield was typically upwards of 98.5% as determined by the dimethoxytrityl cation assay. Sulfurization chemistry utilized the Beaucage reagent. The fully protected monothio RNA combinatorial library with the non-cleavable linker beads were treated with 4 ml of a mixture of 3:1 (v/v) (28%) NH<sub>3</sub>: EtOH at 39

5 °C for 21 hrs. The beads were centrifuged, the supernatant was removed and the solid support was washed with double-distilled water. After lyophilization the solid support was treated with 2 ml of triethylamine trihydrofluoride (TEA-3HF) for 20 hrs at room temperature. Again, the beads were centrifuged, the supernatant was removed and the solid support was washed with double-distilled water.

10 Column 1: 5'-CCTACTCGCGAATTC-  
GA\*UC\*CU\*GA\*AA\*CU\*GU\*UU\*UA\*AG\*GU\*UG\*GC\*CG\*AU\*C-  
GGATCCGGTGGTCTG-Linker-3': (SEQ ID NO:39) **Phosphate**  
Column 2: 5'-CCTACTCGCGAATTC-  
CU\*AG\*GA\*CU\*UG\*GC\*AC\*AA\*CC\*GU\*CA\*CA\*CU\*GC\*UA\*G-  
15 GGATCCGGTGGTCTG-Linker-3' (SEQ ID NO:40) :**MonothioRNA**

#### EXAMPLE VII

NMR spectra of XBY-5 and XBY-15

20 The NOESY, DQCOSY and TOCSY spectra of XBY-2, XBY-6, XBY-5 (200 OD) and XBY-15 (90 OD) have been acquired. NMR structures for XBY-2 and XBY-6 have been determined and shown to differ from the structure of the parent duplex sequence without any dithioate substitutions (Volk, et al., in press). The structures of the other two thioaptamers are being determined.

25 XBY-5: 5'-CC AGGAGAT<sub>S2</sub>T<sub>S2</sub>CCA C-3' (SEQ ID NO:41)  
3'-GG<sub>S2</sub>TCC TCTA A GG<sub>S2</sub>TG-5' (SEQ ID NO:42)

XBY-15: 5'-CC A<sub>S2</sub>G<sub>S2</sub>GA GAT<sub>S2</sub>T<sub>S2</sub>CCAC-3' (SEQ ID NO:43)  
3'-GGT<sub>S2</sub>C<sub>S2</sub>CT<sub>S2</sub>CTA A GGTG-5' (SEQ ID NO:44)

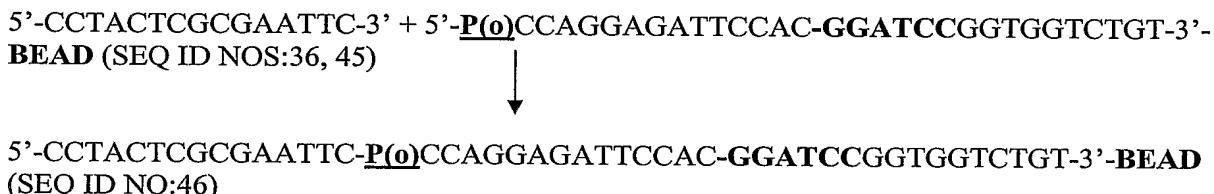
30 EXAMPLE VIII

High quality of one-bead, one-ODN library Ligation Reaction

The present inventors demonstrated that they could construct high quality one-bead one-oligo libraries by join two pieces of DNA based on ligation reaction or highly active phosphorothioate towards 5'-iodo groups on the ODN. Standard phosphoramidite chemistry was used for synthesis of 5' monophosphate ODN (5'-**P(o)**CCAGGAGATTCCAC-**GGATCCGGTGGTCTGT**-bead) (SEQ ID NO:45). The fully protected ODN with the non-cleavable linker beads were treated with concentrated ammonia at 37 °C for 21 hours to remove the protecting groups while

allowing the ODN to remain attached to the beads. A selected single bead was mixed with the following components: 3  $\mu$ l of 40  $\mu$ M 15 mer oligonucleotide (5'-CCTACTCGCGAATTC-3' (SEQ ID NO:36), 3  $\mu$ l of 10 X ligation buffer, 3  $\mu$ l of DMSO, 2  $\mu$ l of T4 RNA ligase and 19  $\mu$ l of ddH<sub>2</sub>O. The reaction was performed at 5 °C for 17 hrs. The supernatant was removed 5 carefully and washed with water. The single bead was performed PCR reaction at established conditions. The PCR products were analyzed on a 15% native polyacrylamide gel. The PCR product was cloned using the TA Cloning procedure (Invitrogen) and sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The desired sequence (5'-CCTACTCGCGAATTC-**P(o)**CCAGGAGATTCCAC-**GGATCCGGTGGTCTGT**-bead) (SEQ 10 ID NO:46), was obtained.

To demonstrate the ligation reaction, a simple ODN was ligated to a single bead of a one-bead, one-ODN library, namely:



20 The ligation reaction was confirmed by one-bead PCR reaction and cloning and sequencing. These results show that the additional nucleic acid sequences may be added to one or more of the beads of a one-bead, one-ODN library with high quality and efficiency while maintaining the integrity of the library. The ligation reaction allows longer random regions of aptamers to be synthesized on the beads with higher yield since a primer region does not have to be stepwise 25 synthesized onto the bead sequence.

#### EXAMPLE IX

##### Separation of Synthetic Oligonucleotide Dithioates from Monothiophosphate Impurities by Anion-exchange Chromatography on a Mono Q Column

30 A method using a strong anion-exchange liquid chromatography column, Mono Q, has been developed for high resolution analysis and purification of oligonucleotide dithioates, which were synthesized by an automated, solid-phase, phosphorothioamidite chemistry. High-resolution separation of oligonucleotide phosphorodithioates from monothiophosphate impurities was obtained. High-resolution separation was also demonstrated at pH 8. The separation of oligonucleotide dithioates was found to be linearly dependent on the number of sulfurs for the

same sequence length. Thiocyanate, SCN<sup>-</sup>, as eluting anion, can be used to purify oligonucleotides containing a high percentage of phosphorodithioate linkages in lower salt concentration, and provide better separation than that of chloride as eluting anion.

**Synthesis of oligomers.** The following oligomers were synthesized for this study:

5 New Scramble : 5'-CCA GT<sub>S2</sub>GA CT<sub>S2</sub>CA GT<sub>S2</sub>G-3' (SEQ ID NO:47)  
3'-GGT<sub>S2</sub>CA CT<sub>S2</sub>GA GT<sub>S2</sub>CA C-5' (SEQ ID NO:48)

5'-amino-xby6: 5'-H<sub>2</sub>NC<sub>12</sub>H<sub>24</sub>-O<sub>3</sub>P-O-CCAGG A GA T<sub>S2</sub>T<sub>S2</sub>CCA C-3' (SEQ ID NO:49)  
3'-GGT<sub>S2</sub>CCT<sub>S2</sub>CT<sub>S2</sub>A A GGT<sub>S2</sub>G-5' (SEQ ID NO:50)

10 5'-fluorescein-xby6: 5'-C<sub>6</sub>H<sub>12</sub>-O<sub>3</sub>P-O-CCA GGA GA T<sub>S2</sub>T<sub>S2</sub>CCA C-3', (SEQ ID NO:51)  
3'-GGT<sub>S2</sub>CCT<sub>S2</sub>CT<sub>S2</sub>A A GGT<sub>S2</sub>G-5' (SEQ ID NO:52)

**XBY-6 and IgkB-22 on Beads for:**  
5'-AGTTGAGGGGACTTCCCAGGCTT-bead (IgkB) (SEQ ID NO:53)

15 3'-TCAACTCCCCTGAAAGGGTCCG-5' (SEQ ID NO:54)

5'-CC AGG AG AT<sub>S2</sub>T<sub>S2</sub>CC AC-linker-bead (XBY-6) (SEQ ID NO:55)  
3'-GG<sub>S2</sub>TCC<sub>S2</sub>TC<sub>S2</sub>T A A GG<sub>S2</sub>TG-5' (SEQ ID NO:56)

20 XBY20-26 for EMSA Competition Assay

XBY-20:	dithioP50-1:	5'-CGC CC <sub>S2</sub> A GTG <sub>S2</sub> As <sub>S2</sub> AG GTG <sub>S2</sub> As <sub>S2</sub> A CCCC-3'	(SEQ ID NO:57)
	dithioP50-1c:	5'-GGG GTT CC <sub>S2</sub> A C CTT C <sub>S2</sub> AC TGG GCG-3'	(SEQ ID NO:58)
XBY-21:	dithioP50-2:	5'-CGC CC <sub>S2</sub> A GTG <sub>S2</sub> AAG GTG <sub>S2</sub> As <sub>S2</sub> A CCCC-3'	(SEQ ID NO:59)
	dithioP50-1c:	5'-GGG GTT CC <sub>S2</sub> A C CTT C <sub>S2</sub> AC TGG GCG-3'	(SEQ ID NO:60)
XBY-22:	dithioP50-3:	5'-CGC CC <sub>S2</sub> A GTGAAG GTG <sub>S2</sub> As <sub>S2</sub> A CCCC-3'	(SEQ ID NO:61)
	dithioP50-1c:	5'-GGG GTT CC <sub>S2</sub> A C CTT C <sub>S2</sub> AC TGG GCG-3'	(SEQ ID NO:62)
XBY-23:	dithioP50-1:	5'-CGC CC <sub>S2</sub> A GTG <sub>S2</sub> As <sub>S2</sub> AG GTG <sub>S2</sub> As <sub>S2</sub> A CCCC-3'	(SEQ ID NO:63)

	phosphateP50-1c:	5'-GGG GTT CCA C CTT C AC TGG GCG-3'	(SEQ ID NO:64)
XBY-24:	dithioP50-2:	5'-CGC CC <sub>S2</sub> A GTG S <sub>2</sub> AAG GTG GA <sub>S2</sub> A CCCC-3'	(SEQ ID NO:65)
	phosphateP50-1c:	5'-GGG GTT CCA C CTT C AC TGG GCG-3'	(SEQ ID NO:66)
XBY-25:	dithioP50-3:	5'-CGC CC <sub>S2</sub> A GTGAAG GTG GA <sub>S2</sub> A CCCC-3'	(SEQ ID NO:67)
	phosphateP50-1c:	5'-GGG GTT CCA C CTT C AC TGG GCG-3'	(SEQ ID NO:68)
XBY-26:	PhosphateP50-1:	5'-CGC CCA GTGAAG GTG GAA CCCC-3'	(SEQ ID NO:69)
	dithioP50-1c:	5'-GGG GTT CC <sub>S2</sub> A C CTT C <sub>S2</sub> AC TGG GCG-3'	(SEQ ID NO:70)

It was found that the XBY20-26 ODN does not compete as well as the selected oligo (monothio selected) with the recombinant NF- $\kappa$ B p50. The chemically synthesized selected oligo (2) was the best so far.

- 5 All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.
- 10 While this invention has been described in reference to illustrative embodiments, this description is not intended to be construed in a limiting sense. Various modifications and combinations of the illustrative embodiments, as well as other embodiments of the invention, will be apparent to persons skilled in the art upon reference to the description.

What is claimed is:

- 1 1. A combinatorial library comprising:
  - 2 two or more beads, wherein attached to each bead is a unique nucleic acid aptamer that
  - 3 comprises a unique sequence.
- 1 2. The library of claim 1, wherein the aptamers are attached covalently to the one or
- 2 more beads.
- 1 3. The library of claim 1, wherein the beads are polystyrene/polydivinyl benzene
- 2 copolymer.
- 1 4. The library of claim 1, wherein the aptamers comprise one or more
- 2 phosphorothioate linkages.
- 1 5. The library of claim 1, wherein the aptamers comprise one or more
- 2 phosphorodithioate linkages.
- 1 6. The library of claim 1, wherein the aptamers comprise one or more
- 2 methylphosphonate linkages.
- 1 7. The library of claim 1, wherein the aptamers further comprise one or more
- 2 predetermined nucleic acid sequences.
- 1 8. The library of claim 1, wherein the aptamers further comprise one or more
- 2 predetermined nucleic acid sequences of at least 10 bases.
- 1 9. The library of claim 1, wherein the aptamer further comprises a nucleic acid
- 2 comprising a predetermined sequence selected from the group consisting of a 5' nucleic acid
- 3 sequence, a 3' nucleic acid sequence, and a 5' and a 3' nucleic acid sequence.
- 1 10. The library of claim 1, wherein the aptamer further comprises a nucleic acid
- 2 comprising a predetermined sequence selected from the group consisting of a 5' nucleic acid

3 sequence, a 3' nucleic acid sequence, and a 5' and a 3' nucleic acid sequence and the  
4 predetermined sequence is synthesized in-line with the aptamer.

1 11. The library of claim 1, wherein the library comprises a S-ODN library.

1 12. The library of claim 1, wherein the library comprises an S<sub>2</sub>-ODN library.

1 13. The library of claim 1, wherein the aptamers are attached to the beads by a  
2 hexaethyleneglycol linker.

1 14. The library of claim 1, wherein the aptamer comprises a nuclear protein binding  
2 site sequence.

1 15. The library of claim 1, wherein the aptamers are isosteric and isopolar.

1 16. The library of claim 1, wherein the aptamers are achiral.

1 17. The library of claim 1, wherein the aptamer is a XBY-6-like library.

1 18. The library of claim 1, wherein each of the aptamers are the same length.

1 19. The library of claim 1, wherein each of the aptamers is further modified to  
2 comprise a colorimetric agent.

1 20. The library of claim 1, wherein each of the aptamers further comprises one or  
2 more bases that are attached to a fluorophor.

1 21. The library of claim 1, wherein each of the aptamers further comprises one or  
2 more fluorophors attached to the 5' end, the 3' end or internally within the aptamers.

1 22. The library of claim 1, further comprising the complementary strand to the  
2 aptamer.

1 23. The library of claim 1, wherein the library is double-stranded.

1 24. The library of claim 1, wherein the library is a peptide nucleic acid library.

1           25.    The library of claim 1, wherein the aptamer comprises both modified and  
2    unmodified nucleotides.

1           26.    The library of claim 1, wherein the aptamer comprises both modified and  
2    unmodified nucleotides and the aptamer is partially thio-phosphate modified.

1           27.    The library of claim 1, wherein the complementary strand of the modified  
2    nucleotide aptamer is selected from the group consisting of dATP( $\alpha$ S), dTTP( $\alpha$ S), dCTP( $\alpha$ S)  
3    and dGTP( $\alpha$ S).

1           28.    The library of claim 1, wherein the aptamer is selected by amplifying the library  
2    enzymatically using a mix of four nucleotides, wherein at least a portion of at least one and no  
3    more than three of the nucleotides in the mix is thiophosphate-modified, to form a partially  
4    thiophosphate-modified oligonucleotide combinatorial library.

1           29.    The library of claim 1, wherein no more than three adjacent phosphate sites of the  
2    aptamer are replaced with phosphorothioate groups.

1           30.    The library of claim 1, wherein the aptamer is created by a split and pool  
2    combinatorial synthesis chemistry.

1           31.    The library of claim 1, wherein at least a portion of non-adjacent dA, dC, dG, or  
2    dT phosphate sites of the aptamer are replaced with phosphorothioate groups.

1           32.    The library of claim 1, wherein all of the non-adjacent dA, dC, dG, or dT  
2    phosphate sites of the aptamer are replaced with phosphorodithioate groups.

1           33.    The library of claim 1, wherein substantially all non-adjacent phosphate sites of  
2    the aptamer are replaced with phosphorothioate groups.

1           34.    The library of claim 1, wherein no more than three adjacent phosphate sites of the  
2    aptamer are replaced with phosphorodithioate groups.

1           35.    A one-bead, one-ODN combinatorial library comprising:

2           two or more beads, wherein attached to each bead is a unique aptamer comprising a  
3       single unique sequence, wherein each unique aptamer comprises a mix of modified and  
4       unmodified nucleotides.

1           36.    A combinatorial library comprising:

2           two or more beads, and

3           a unique nucleic acid aptamer that comprises a single unique sequence attached to each of  
4       the two or more beads, and

5           a complementary strand attached to the unique nucleic acid, wherein each bead comprises  
6       a unique double-stranded aptamer.

1           37.    An ODN library comprising:

2           a library substrate comprising a surface; and

3           a one-bead, one-ODN bead library attached to the library substrate surface.

4           The ODN library of claim 37, wherein the library substrate comprises a set of beads.

1           38.    The ODN library of claim 37, wherein the library substrate comprises further a  
2       chip.

1           39.    The ODN library of claim 37, wherein the library substrate comprises further a  
2       chip that comprises a capacitance coupled detector.

1           40.    The ODN library of claim 37, wherein the library substrate comprises a  
2       photolithographically etched micromachined microwell chip.

1           41.    The ODN library of claim 37, wherein the library substrate comprises a glass  
2       slide.

1           42.    A method of making a combinatorial library comprising the steps of:

2           attaching a single base to a first set of beads in a first column and a single thio-modified  
3       base to a second set of beads in a second column;

- 4 mixing the first and second set of beads;
- 5 splitting the mixed first and second set of beads into the first and second columns;
- 6 adding a new base to the mixed beads in each of the first and second columns; and
- 7 repeating the steps of mixing the beads, splitting the beads and adding a new base in each
- 8 of the first and second columns until the library is complete.

1           43.     The method of claim 42, further comprising the step of synthesizing a  
2 predetermined sequence in the first and second columns in parallel.

1                   44.     The method of claim 42, wherein the aptamers are attached to the column from  
2     the 3' end.

1           45. The method of claim 42, further comprising the step of converting the aptamer to  
2 a double-stranded aptamer with a DNA polymerase I Klenow fragment.

1           46.     The method of claim 42, further comprising the step of isolating one or more  
2     beads with a bound molecule by micromanipulation.

1           47.     The method of claim 42, wherein the aptamers further comprise one or more  
2     bases that are attached to a fluorophor.

1           48.     The method of claim 42, wherein the aptamers further comprise one or more  
2     fluorophors attached to the 5' end, the 3' end or internally within the aptamers

1 49. A method of making a one-bead, one-ODN combinatorial library comprising the  
2 steps of:

3 attaching a single base to the first beads in a first column;

4 attaching a single modified base or phosphate backbone analog to the second set of beads  
5 in a second column:

6 mixing the first and second set of beads from the first and second columns;

7                   splitting the mixed first and second set of beads into the first and second columns;

8                   adding a new base to the mixed beads in each of the first and second columns; and

9                   repeating the steps of mixing the beads, splitting the beads and adding a new base in each

10                  of the first and second columns until the library is complete, wherein each of the

11                  oligonucleotides on each bead is unique and each unique oligonucleotide on each bead comprises

12                  a mix of modified and unmodified nucleotides.

1                50. A method for detecting nucleic acid-protein interactions comprising the steps of:

2                   mixing a one-bead, one-ODN combinatorial library with one or more proteins; and

3                   detecting the binding of the protein to one or more beads of the one-bead, one-ODN

4                  combinatorial library.

1                51.        The method of claim 50, wherein the step of mixing the one-bead, one-ODN

2                  combinatorial library with one or more proteins is under conditions that permit the binding of a

3                  second protein to the protein bound to the one or more beads.

1                52.        The method of claim 50, further comprising the step of identifying the sequence

2                  on the bead by isolating the bead and sequencing the unique aptamer bound to the bead.

1                53.        The method of claim 50, further comprising the step of identifying the sequence

2                  on the bead by isolating the bead and sequencing the unique aptamer bound to the bead by PCR.

1                54.        The method of claim 50, further comprising the step of identifying the sequence

2                  on the bead by isolating the bead and sequencing the unique aptamer bound to the bead by PCR

3                  tagging.

1                55.        The method of claim 50, wherein the ODNs further comprise one or more bases

2                  that are attached to a fluorophor.

1                56.        The method of claim 50, wherein the ODNs further comprise one or more

2                  fluorophors attached to the 5' end, the 3' end or internally within the aptamers.

1           57.    The method of claim 50, further comprising the step of comparing the level of  
2 protein bound to the one or more beads between one or more proteins from a control and a test  
3 sample.

1           58.    The method of claim 50, further comprising the step of comparing the level of  
2 protein bound to the one or more beads between one or more proteins from a control and a  
3 patient sample.

1           59.    A method to identify proteins differentially expressed in a sample, comprising the  
2 steps of:

3           mixing a one-bead, one-ODN aptamer bead library comprising two or more beads to a  
4 sample, wherein the sample has been labeled with a first dye and a control, wherein the control  
5 has been labeled with a second dye under conditions that allow binding; and

6           sorting the bead library by comparing the relative levels of each of the first and second  
7 dyes on each bead, wherein differences in the level of the first or second dye are used to  
8 determine the level of binding of the sample and the control to the beads in the bead library.

1           60.    The method of claim 59, wherein the beads are sorted by a flow cytometer.

1           61.    The method of claim 59, wherein the beads are sorted manually.

1           62.    The method of claim 59, further comprising the step of isolating the one or more  
2 beads.

1           63.    The method of claim 59, further comprising the step of isolating the one or more  
2 beads and determining the characteristics of the bound material by SELDI-MS.

1           64.    The method of claim 59, wherein the first and second dyes are fluorescent dyes.

1           65.    The method of claim 59, wherein the first and second dyes are cy3 and cy5.

1           66.    The method of claim 59, wherein the one or more beads are isolated and placed  
2 onto a substrate.

1        67. The method of claim 59, wherein the ODNs further comprise one or more bases  
2 that are attached to a fluorophor.

1        68. The method of claim 59, wherein the ODNs further comprise one or more  
2 fluorophors attached to the 5' end, the 3' end or internally within the aptamers.

1        69. The method of claim 59, wherein the ODNs further comprise multiple dyes  
2 attached covalently to the ODNs.

1        70. The method of claim 59, wherein the bead library is placed on a Texas tongue.

1        71. The method of claim 59, wherein the ODNs comprise two or more dyes and  
2 further comprising the step of sorting the beads in the bead library by a low, medium and/or high  
3 selection criteria.

1        72. A method of making a library of random combinatorial library beads comprising  
2 the steps of:

3        attaching a single base to a first set of beads in a first column;

4        attaching a mixture of unmodified and modified bases to a second set of beads in a  
5 second column;

6        mixing the first and second set of beads from the first and second columns;

7        splitting the mixed first and second set of beads into the first and second columns;

8        adding a new base selected from the group consisting of unmodified bases, modified  
9 bases, phosphate backbone analogs and mixtures thereof to the mixed beads in each of the first  
10 and second columns; and

11        repeating the steps of mixing the beads, splitting the beads and adding a new in each of  
12 the first and second columns until the library is complete, wherein each of the oligonucleotides  
13 on each bead is a random combinatorial library of base or backbone modifications at selected  
14 sites.

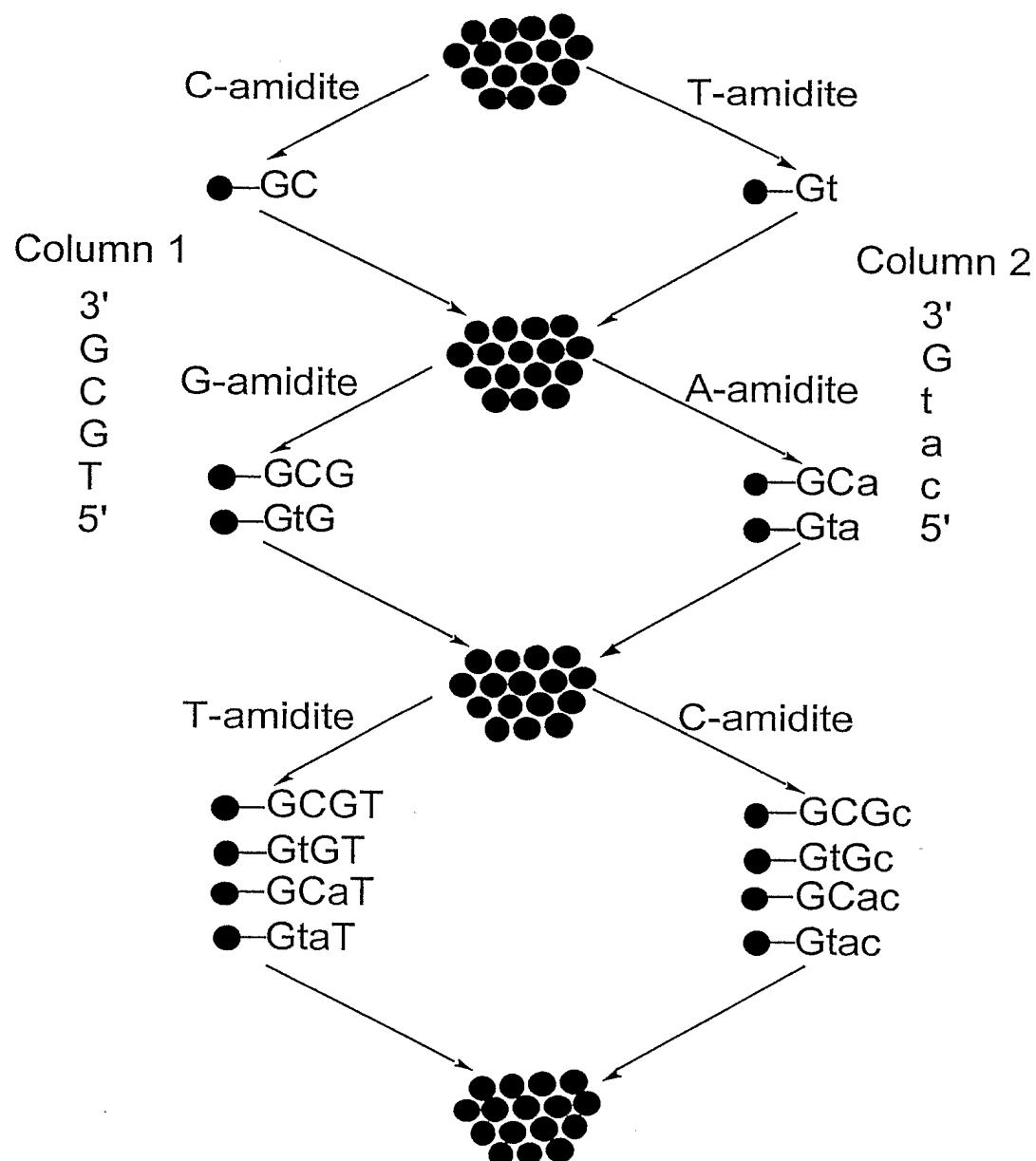


FIGURE 1

Figure 2

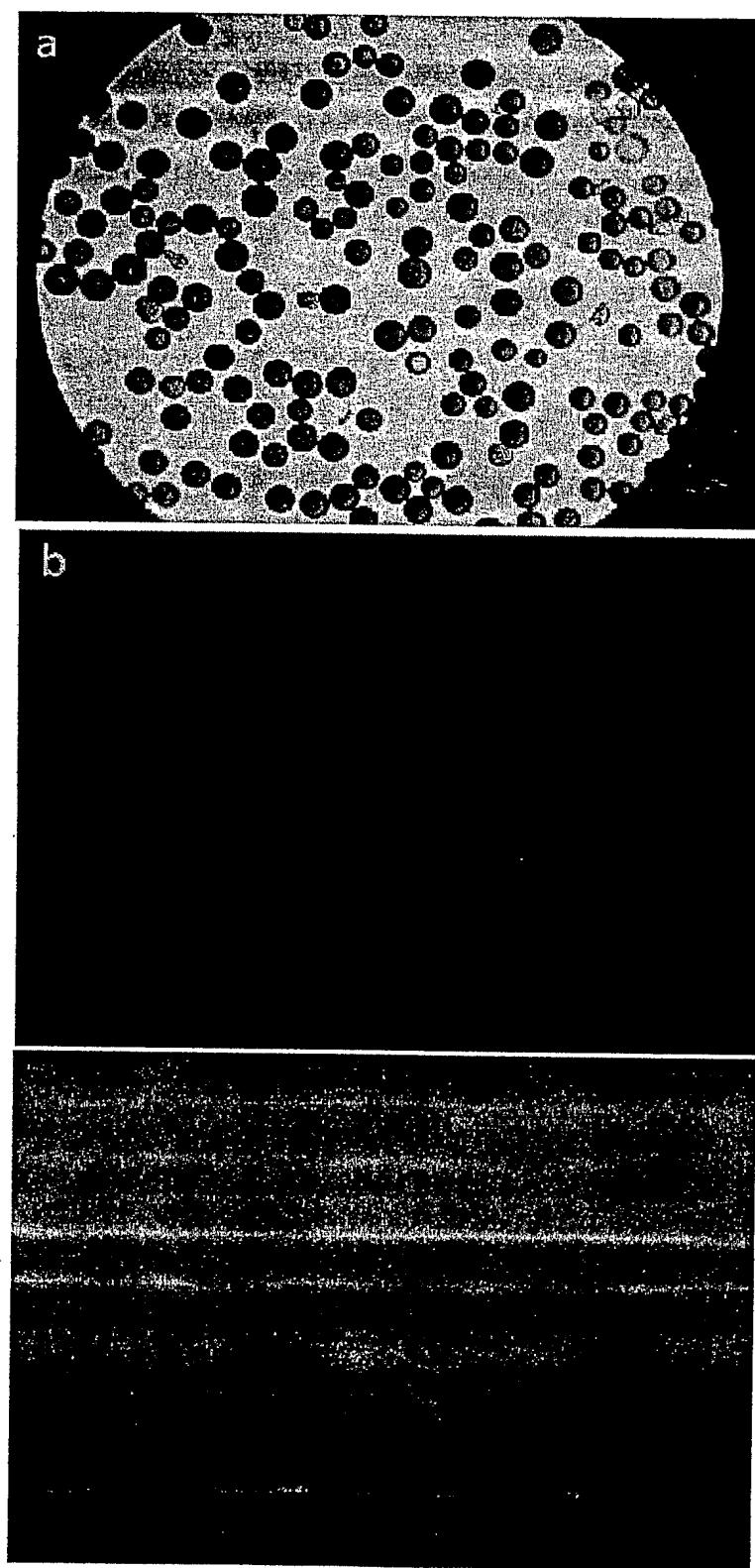


Figure 3A

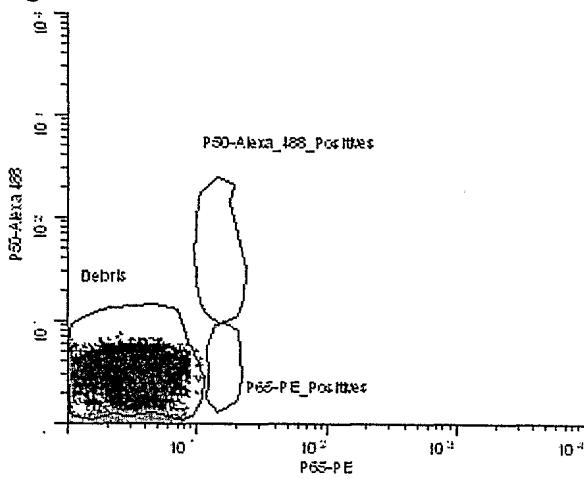


Figure 3B

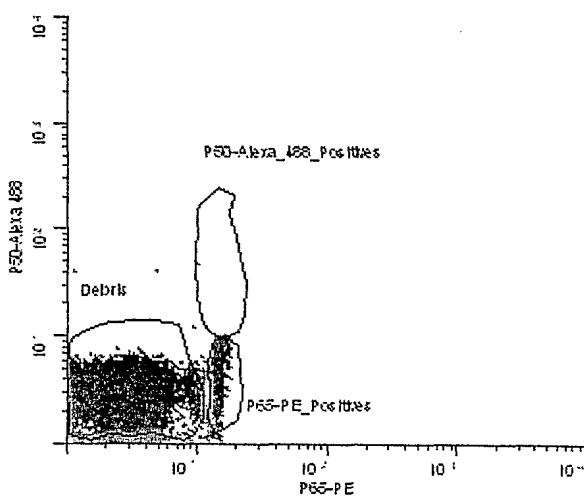
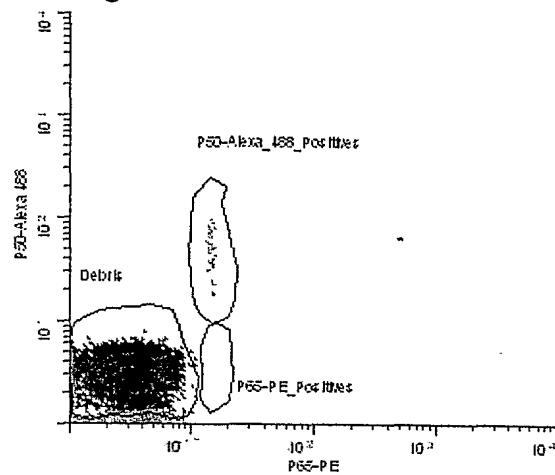


Figure 3C

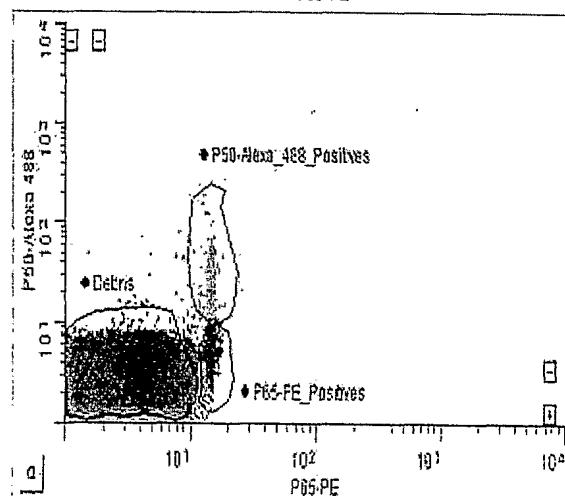


Figure 3D

## SEQUENCE LISTING

<110> Board of Regents, The University of Texas System  
Gorenstein, David G.  
Luxon, Bruce A.  
Herzog, Norbert  
Tang, Xian B.

<120> BEAD BOUND COMBINATORIAL OLIGONUCLEOSIDE PHOSPHOROTHIOATE AND  
PHOSPHORODITHIOATE APTAMER LIBRARIES

<130> UTMB:1013

<140> 10/272,509  
<141> 2002-10-16

<160> 70

<170> PatentIn version 3.2

<210> 1  
<211> 15  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 1  
ggatccggtg gtctg 15

<210> 2  
<211> 15  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 2  
cctactcgcg aattc 15

<210> 3  
<211> 23  
<212> DNA  
<213> artificial

<220>  
<223> wherein at least one nucleotide is an achiral thiophosphate or

dithiophosphate

<220>  
<221> modified\_base  
<222> (1)..(23)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23.

<400> 3  
cagttgaggg gactttccca ggc

23

<210> 4  
<211> 23  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(23)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23.

<400> 4  
cctgcacatc tcaggatgac ttt

23

<210> 5  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 4, 8, 9, 16, 19.

<400> 5  
atgtagccag ctagtctgtc ag

22

<210> 6  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 5, 9, 10, 16, 19.

<400> 6  
cgcccagtga aggttggaaacc cc

22

<210> 7  
<211> 50  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 7  
atgcctactc gcgaattccc aggagattcc acggatccgg tggctgttc

50

<210> 8  
<211> 52  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 8  
cctactcgcg aattcagttg aggggacttt cccaggcgga tccgggtggtc tg

52

<210> 9  
<211> 18  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: consensus sequence

<400> 9  
atgcctactc gcgaattc

18

```
<210> 10
<211> 18
<212> DNA
<213> Artificial

<220>
<223> Synthetic oligonucleotide.

<220>
<221> misc_feature
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 10
gaacagacca ccggatcc                                18

<210> 11
<211> 22
<212> DNA
<213> Artificial

<220>
<223> Synthetic oligonucleotide.

<220>
<221> misc_feature
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 11
ctgtgagtcg actgatgacg gt                                22

<210> 12
<211> 22
<212> DNA
<213> Artificial

<220>
<223> Synthetic oligonucleotide.

<220>
<221> misc_feature
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 12
agttgagtcg aaggacccat tt                                22

<210> 13
<211> 22
<212> DNA
<213> Artificial

<220>
<223> Synthetic oligonucleotide.
```

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 13  
cgtcaagtct cagttcccat tt

22

<210> 14  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 14  
agtcaagtgc aagttccacg gt

22

<210> 15  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 2, 8, 14, 16, 22.

<400> 15  
ctgtgagtcg actgatgacg gt

22

<210> 16  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 8, 12, 14, 20, 22.

<400> 16  
agtgtgagtcg aaggacccat tt

22

<210> 17  
<211> 22  
<212> DNA  
<213> Artificial  
  
<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 4, 8, 10, 12, 20, 22.  
  
<400> 17  
cgtcaagtct cagttccat tt

22

<210> 18  
<211> 22  
<212> DNA  
<213> Artificial  
  
<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 4, 8, 12, 18, 22.  
  
<400> 18  
agtcaagtcg aagttccacg gt

22

<210> 19  
<211> 22  
<212> DNA  
<213> Artificial  
  
<220>  
<223> Artificial oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide  
  
<400> 19  
atgttagccag ctagtctgtc ag

22

<210> 20  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 20  
cgccagccaa aggtgctgtc ag 22

<210> 21  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 21  
cggccagtggtt ctagtgaacc cc 22

<210> 22  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 22  
atgttagccga aggttggaaacc cc 22

<210> 23  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 23

cggccagccga aggttggacc cc

22

<210> 24  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 24  
atgttagccag ctagtctgtc ag

22

<210> 25  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 10.

<400> 25  
cgccagccaa aggtgctgtc ag

22

<210> 26  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 5, 9, 16, 17.

<400> 26  
cgcccagtggtt ctagtgaacc cc

22

<210> 27  
<211> 22  
<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>

<221> modified\_base

<222> (1)..(22)

<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 9, 10, 16, 17.

<400> 27

atgttagccga aggttggaaacc cc

22

<210> 28

<211> 22

<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>

<221> modified\_base

<222> (1)..(22)

<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 10, 11, 17, 18.

<400> 28

cggccagccgaa aggttggaaacc cc

22

<210> 29

<211> 22

<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>

<221> misc\_feature

<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 29

gggggttccac cttcactggg cg

22

<210> 30

<211> 22

<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 30  
ccccaaagggtg gaagtgaccc gc

22

<210> 31  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 10.

<400> 31  
cgccagccga aggtgctgtc ag

22

<210> 32  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 10, 16, 17.

<400> 32  
atgttagccaa aggtggaacc cc

22

<210> 33  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 5, 9, 10.

<400> 33  
cgccccagtga aggtgctgtc ag 22

<210> 34  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 4.

<400> 34  
cgccccagttag ctagtctgtc ag 22

<210> 35  
<211> 15  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 35  
ggatccgggtg gtctg 15

<210> 36  
<211> 15  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 36  
cctactcgcg aattc 15

<210> 37  
<211> 14  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.  
  
<220>  
<221> modified\_base  
<222> (1)..(14)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 9, 10.

<400> 37  
ccaggagatt ccac

14

<210> 38  
<211> 14  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(14)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 3, 13.

<400> 38  
gtggaatctc ctgg

14

<210> 39  
<211> 14  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(14)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 4, 5, 10, 11.

<400> 39  
ccaggagatt ccac

14

<210> 40  
<211> 14  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(14)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 9, 11, 12.

<400> 40  
gtggaatcyc cygg

14

<210> 41  
<211> 30  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(30)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at position 1.

<400> 41  
ccaggagatt ccacggatcc ggtggctgt

30

<210> 42  
<211> 45  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (16)..(16)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate.

<400> 42  
cctactcgcg aattccagg agattccacg gatccggtgg tctgt

45

<210> 43  
<211> 14  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(14)

<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 5, 9, 13.

<400> 43

ccagtgactc agtg

14

<210> 44

<211> 14

<212> DNA

<213> Artificial

<220>

<223> modified\_base

<222> (1)..(14)

<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 4, 8, 12,

<400> 44

ggtcactgag tcac

14

<210> 45

<211> 14

<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>

<223> modified\_base

<222> (1)..(14)

<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 10, 11.

<400> 45

ccaggagatt ccac

14

<210> 46

<211> 14

<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>

<223> modified\_base

<222> (1)..(14)

<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 4, 7, 9, 14.

<400> 46

ggtcctctaa ggtg

14

<210> 47  
<211> 14  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(14)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 10, 11.

<400> 47  
ccaggagatt ccac

14

<210> 48  
<211> 14  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(14)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 4, 7, 9, 14.

<400> 48  
ggtcctctaa ggtg

14

<210> 49  
<211> 24  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of artificial sequence: synthetic oligonucleotide

<400> 49  
agttgagggg actttccca gctt

24

<210> 50  
<211> 22  
<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>

<221> misc\_feature

<223> Description of artificial sequence: synthetic oligonucleotide

<400> 50

gcctggaaa gtcccccaa ct

22

<210> 51

<211> 14

<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>

<221> modified\_base

<222> (1)..(14)

<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 10, 11.

<400> 51

ccaggagatt ccac

14

<210> 52

<211> 14

<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>

<221> modified\_base

<222> (1)..(14)

<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 3, 7, 9, 13.

<400> 52

gtggaaatctc ctgg

14

<210> 53

<211> 22

<212> DNA

<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 6, 10, 11, 17, 18

<400> 53  
cgccccagtga aggttggAACCC

22

<210> 54  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 9, 15.

<400> 54  
ggggttccac cttcactggg cg

22

<210> 55  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 6, 10, 18.

<400> 55  
cgccccagtga aggttggAACCC

22

<210> 56  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or

dithiophosphate at positions 9, 15.

<400> 56  
ggggttccac cttcaactggg cg

22

<210> 57  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 6, 18.

<400> 57  
cgcccagtga aggttggacc cc

22

<210> 58  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 9, 15.

<400> 58  
ggggttccac cttcaactggg cg

22

<210> 59  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 6, 11, 12, 18, 19.

<400> 59  
cgcccagtga aggttggacc cc

22

<210> 60  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of artificial sequence: synthetic oligonucleotide

<400> 60  
ggggttccac cttcactggg cg 22

<210> 61  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 6, 10, 18.

<400> 61  
cgcccagtga aggtggAACCC 22

<210> 62  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of artificial sequence: synthetic oligonucleotide

<400> 62  
ggggttccac cttcactggg cg 22

<210> 63  
<211> 22  
<212> DNA  
<213> Artificial

<220>

<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 6, 18.

<400> 63  
cgccccagtga aggttggacc cc

22

<210> 64  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of artificial sequence: synthetic oligonucleotide

<400> 64  
ggggttccac cttcactggg cg

22

<210> 65  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> misc\_feature  
<223> Description of artificial sequence: synthetic oligonucleotide

<400> 65  
cgccccagtga aggttggacc cc

22

<210> 66  
<211> 22  
<212> DNA  
<213> Artificial

<220>  
<223> Synthetic oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(22)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 9, 15.

<400> 66  
ggggttccac cttcactggg cg 22

<210> 67  
<211> 31  
<212> RNA  
<213> Artificial

<220>  
<223> Artificial oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(31)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 19, 31, 33.

<400> 67  
gauccugaaa cuguuuuaag guuggccgau c 31

<210> 68  
<211> 31  
<212> RNA  
<213> Artificial

<220>  
<223> Artificial oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(31)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31.

<400> 68  
cuaggacuug gcacaaccgu cacacugcua u 31

<210> 69  
<211> 61  
<212> DNA  
<213> Artificial

<220>  
<223> Artificial oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(61)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31.

<400> 69  
cctactcgcg aattccuagg acuuggcaca accgucacac ugcuagggat ccggtggtct 60  
g 61

<210> 70  
<211> 61  
<212> DNA  
<213> Artificial

<220>  
<223> Artificial oligonucleotide.

<220>  
<221> modified\_base  
<222> (1)..(61)  
<223> wherein at least one nucleotide is an achiral thiophosphate or dithiophosphate at positions 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31.

<400> 70  
cctactcgcg aattcgaucc ugaaacuguu uuaagguugg ccgaucggat ccggtggtct 60  
g 61